GENETICS OF PROTOZOA ESPECIALLY IN RELATION TO MITOCHONDRIA

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SUMMARY

1. A technique for microinjecting individual paramecium cells with small volumes of liquid has been developed. Using this technique it was shown that cytoplasmically inherited erythromycin resistance in Paramecium aurelia was ~~treated~~ located in the mitochondria, probably on the mitochondrial DNA. The transmission of this erythromycin resistance by microinjection was shown to be completely removed by low concentrations of non ionic detergent while remaining unaffected by DNAse or RNAse.

2. Preliminary evidence is presented concerned with the number of mitochondria required to transmit erythromycin resistance to a sensitive cell. A statistical analysis of these results suggests that only one or two mitochondria may be sufficient to transfer resistance.

3. The effect of erythromycin on the mitochondrial morphology of both sensitive and resistant cells was studied. Electron micrographs of cells exposed to erythromycin for various lengths of time showed that the antibiotic has striking effects on mitochondrial structure: the cristae were lost progressively with time of exposure and new structures were seen to develop.

4. The change from sensitivity to erythromycin resistance following microinjection was studied by electronmicroscope observations of individually fixed cells. The existence of two

distinct classes of mitochondria in these transforming cells suggests that mitochondria are autonomous with respect to erythromycin resistance. This evidence implies that erythromycin resistance is acquired by direct selection for the newly introduced "resistant" mitochondria.

5. Erythromycin resistant mitochondria have been transferred by microinjection between three different syngens of Paramecium aurelia. It was found that the proportion of cells developing resistance in certain intersyngen transfers was much lower than in intra-syngen transfers using the same mitochondrial preparation.

6. Two classes of lowered transmission of erythromycin resistance were noted. The first class, illustrated by reciprocal transfers between syngens 1 and 5, was shown to be due to a modification of the mitochondria by the nucleus of syngen 1 such that the organelles became altered in their ability to transmit erythromycin resistance to syngen 5. The second class, illustrated by transfers between syngens 5 and 7, was shown to be caused by the different syngenic origins of the mitochondrial genomes used.

7. "Hybrid" cells containing mitochondria derived from one syngen by microinjection into the cytoplasm of another were used to show that the mitochondrial enzyme fumerase is coded for by the nuclear genome.

8. An account of the possible approaches for characterizing specific proteins determined by the mitochondria has been given. The "hybrid" cells obtained in this work were considered important as they offer a unique tool for the identification of proteins coded for by the mitochondrial DNA.

GENERAL INTRODUCTION

One of the characteristic features of eukaryotic cells is that they all contain mitochondria, the membrane bound organelles in which most cellular respiration is localised. The existence of various types of cytoplasmic inheritance had been accepted for many years but the demonstration by Nass, Nass and Afzelius (1965) that mitochondria from many organisms contained small amounts of DNA gave a new impetus to the study of the biochemical genetics of mitochondria.

It is now certain that the information encoded on the mitochondrial DNA is an essential part of the total inheritance of the eukaryotic cell. However, the exact nature of this information is less certain.

In recent years, the greatest advances in our knowledge of the genetic system of mitochondria have come through two types of approach.

1. Biochemical examination of the properties of mitochondrial nucleic acids, particularly in the laboratories of Borst and Attardi, has set limits on the extent and nature of the information coded by mitochondrial DNA.

2. Studies of recombination between antibiotic resistant markers in yeast, initiated by Linnane and his co-workers (Linnane et al, 1968a and b) and continued in the laboratories of Wilkie and Slonimski, have provided much information about the nature of mitochondrial genes in yeast.

Mitochondrial nucleic acids have been recently reviewed in detail by Borst (1972) and so only a brief summary will be presented here. Mitochondrial DNAs appear to be usually circular, and often supercoiled molecules of defined length. There does not appear to be any relationship between the (GC) content of mitochondrial and their respective nuclear DNAs.

The information content of mitochondrial DNAs has been studied by quantitative renaturation experiments. Most mitochondrial DNAs studied follow ideal second order renaturation kinetics and so large scale heterogeneity is ruled out. Experiments with several organisms involving hybridization of labelled ribosomal RNA to mitochondrial DNA has shown that each DNA molecule probably contains only one copy of each of the mitochondrial ribosomal RNAs. Similar experiments using transfer RNAs charged with specific amino acids have shown that mitochondrial DNAs code for between four and eleven transfer RNAs. It therefore seems that either some tRNAs must be imported into the mitochondria or that proteins synthesised on mitochondrial ribosomes must have an unusual amino acid composition.

Our knowledge of the proteins coded for by the mitochondrial DNA is still incomplete because definitive identification of these products requires the correlation of changes in mitochondrial proteins with changes in mitochondrial DNAs, something that has proved difficult until the present time.

The following four approaches have been used generally in attempts to investigate this problem (from Borst, 1972).

1. Pulse labeling with amino acids in the presence of specific inhibitors of mitochondrial or nucleo-cell-sap transcription or translation.

2. Identification of the products of protein synthesis by isolated mitochondria.

3. Identification of mitochondrial proteins that are not specified by mitochondrial genes because they can still be synthesized in the absence of functional mitochondrial DNA.

4. Identification of the altered gene product in mutants that show cytoplasmic inheritance.

Because of the interdependance of nucleo-cytoplasmic and mitochondrial contributions to mitochondrial biogenesis and the uncertainty about possible import or export of messenger RNA, results obtained from the first three methods should be viewed with some reserve.

However, it does seem clear that, whatever the origin of the information, the products of mitochondrial protein synthesis are mainly hydrophobic proteins located in the inner membranes of the mitochondria, some of which may be closely associated with respiratory enzymes.

In conclusion, while there is much circumstantial evidence to show that mitochondria DNA does code for proteins having important functions in mitochondrial biogenesis, many specific questions remain unanswered.

Recent studies on the genetics of mitochondria, particularly in yeast, have demonstrated that mitochondria are capable of complex genetic reorganisation. Thomas and Wilkie (1968) were able to demonstrate independent assortment of the mitochondrial genes coding for chloramphenicol and erythromycin resistance. By the analysis of large numbers of multifactorial crosses Slonimski and his group (Coen et al., 1969; Bolotin et al., 1970) have been able to analyse in detail the nature of sexuality in yeast mitochondria. In heterosexual crosses a polarity of transmission of markers is exhibited while in homosexual crosses

no such polarity of transmission is observed. However, yeast remains the only organism in which it has been possible to demonstrate recombination of mitochondrial genes.

Paramecium aurelia has several unique advantages for the study of mitochondrial genetics (Beale, 1954 and 1969).

1. The genetic properties of this organism make it suitable for the analysis of cytoplasmic mutants and also for the study of nuclear genes found to affect these mutants.

2. Paramecium contains large numbers of mitochondria and biochemical preparations of these are easily obtained.

3. The large size of the cells has allowed firstly, the development of the technique of microinjection, and secondly, the study of quantitative mitochondrial genetics often not possible in many other micro-organisms.

In general, the work presented in this study is concerned with the development of the technique of microinjection which was subsequently used to locate cytoplasmic erythromycin resistance in the mitochondria of P.aurelia. The technique of microinjection has also allowed the transfer of mitochondria from one syngen to others. During the course of this work several interesting interactions between the nucleo-cytoplasmic system and the mitochondrial erythromycin resistance were observed.

MATERIALS AND METHODS

Section I : General Methods

1. Biochemical Reagents

Inorganic chemicals, sucrose, and rafinose were obtained from British Drug Houses Limited and Fisons Limited. Biochemical reagents and enzymes were obtained from Sigma Chemical Company Limited, London. The erythromycin used was a gift from Dr. P. Slonimski. The radioactive ^{14}C DNA prepared from E.coli was a gift from Dr. J. Bishop and the radioactive ^{14}C RNA prepared from Xenopus laevis was a gift from Dr. Mary Lou Pardue.

2. Strains

(a) Several wild type erythromycin sensitive strains of Paramecium aurelia were used in this study. These were as follows:

168 ES	Syngen 1	designated	1(168)ES
513 ES	"	"	1(513)ES
92 ES	Syngen 3	"	3ES
51 ES	Syngen 4	"	4ES
87 ES	Syngen 5	"	5ES
227 ES	Syngen 7	"	7ES
214 ES	Syngen 8	"	8ES
328 ES	Syngen 14	"	14ES

The growth of these stocks is inhibited by 100 µg/ml erythromycin.

(b) Resistant strains.

Spontaneously occurring erythromycin resistant strains have been isolated from some of the wild type stocks listed above. Two of these $168ER_1$ and $87ER_1$ were used in this study. Both of these strains are resistant to 250 µg/ml erythromycin and are designated 1(168)ER and 5 ER respectively. The genetic analysis of these has been described (Beale, 1969; Beale et al., 1972).

(c) "Hybrid" strains

Erythromycin resistant "hybrid" strains are stocks derived from a cell injected with mitochondria from another syngen. Several classes of these were produced during this study. These were as follows:

Symbolic name	mitochondria	Recipient
5 ER m 1.	1 ER	5ES
7 ER m 1.	1 ER	7ES
7 ER m 5.	5 ER	7ES
1 ER m 1.5.	5 ER m 1.	1ES
7 ER m 1.5.	5 ER m 1.	7ES
5 ER m 1.5.1.	1 ER m 1.5.	5ES

Nomenclature of Strains

Each strain has a stock number derived from the isolation number assigned when the original clone was isolated from a natural population. This is followed by either the letters ER signifying resistance to erythromycin, or ES signifying natural sensitivity to 100 µg/ml erythromycin. A subscript number following this is the

isolation number of the mutant strain. "Hybrid" stock names have the subscript 'm' followed by the number of the syngens into which the erythromycin resistance had previously been injected. For example, 7 $ER_{m\ 1.5}$ contains mitochondria originally isolated from syngen 1, injected with syngen 5, reisolated and injected with syngen 7.

3. Culture Media

(a) Concentrated Grass Infusion

1200 grams of dried grass was boiled in 10 litres of distilled water for ten minutes, stirring continuously. After cooling, the suspension was filtered once through four layers of butter muslin. The filtrate was passed through an M.S.E. continuous flow centrifuge head spinning at 15,000 r.p.m., at a flow rate of 200 ml. per minute. The effluent was collected in Thomson bottles and autoclaved for 20 minutes at 20 pounds per square inch. This solution was used for making up growth media as follows:

(b) Mass Culture Medium

500 mls. of the concentrated grass infusion was diluted with 350 ml. of $^{M}/_{5}$ $Na_2H\ PO_4$ and made up to 10 litres with distilled water. One litre aliquots were placed in Thomson bottles and autoclaved ready for use.

(c) Slide medium

50 mls. of concentrated grass infusion were diluted with 70 ml. of $^{M}/_{5}$ $Na_2H\ PO_4$ and made up to two litres with distilled water. 400 ml. aliquots were placed in 500 ml. flasks and autoclaved ready for use. All culture of Paramecium was carried out using bacterized medium. The medium

was bacterized by inoculation with Klebsiella aerogenes cultured on agar slants and incubated for 12 to 18 hours at 31°C. The bacterized medium was adjusted to pH 6.8 to 7.0 with 2 Normal Sodium Hydroxide immediately before use.

4. Culture of Cells

A detailed description of methods used for the culture of paramecia is given by Sonneborn (1950,1971). A brief outline is given here as some of the methods used differ from those previously described.

(a) Slide and Tube Cultures

Selection for erythromycin resistance following microinjection and routine maintenance of stocks was carried out using bacterized slide medium. Erythromycin was made up in a stock solution of 0.5 grams in 20 mls. of analar ethanol. This stock solution was diluted one hundred fold with bacterized slide medium to give a final concentration of 250 µg/ml. in the selective medium. Sensitive cells which had been microinjected with cell extracts were washed once and placed in selective medium at 24°C after being left overnight at 18°C to recover from the "operation".

Strains which were to be used for microinjection were grown in tubes at 18°C and were fed weekly in order to maintain them in the best physiological condition.

(b) Mass Cultures

Cells in 20 ml of slide medium were inoculated into 500 mls. of freshly bacterized slide medium and incubated at 31°C until the

culture cleared as the paramecia ingested the bacteria. The cells were inoculated into 1 litre of bacterized mass culture medium and this medium allowed to clear. This one litre of cells then served to subinoculate up to 8 litres of bacterized mass culture media. The cells were grown up at 24°C. When the paramecia had cleared the bacteria, the culture was ready for harvesting.

5. Harvesting of Paramecia.

Mass cultures were first filtered through a layer of absorbent cotton wool to remove debris from the culture. The filtrate was passed through an M.S.E. continuous flow centrifuge head spinning at 3,000 r.p.m. at a flow rate of 800 ml. per minute. The cells were resuspended in approximately 300 ml. of Dryl's medium (Dryl, 1965) and recentrifuged in an M.S.E. oil testing centrifuge. The pelleted cells were resuspended, pooled and washed twice with Dryl's medium to remove as many bacteria as possible.

6. Preparation of Cell Extracts

The cell extracts prepared for microinjection were of four types; the first three were prepared by differential centrifugation and the last by a sucrose one step gradient.

(a) 500 g. Supernatant

Between 2 and 4 mls. of packed cells were diluted with 10 volumes of raffinose homogenization buffer containing 0.215 M raffinose, 2.5 mg/ml bovine serum albumin and 0.01 M potassium phosphate, pH 6.2 (Preer and Preer, 1959). The resulting suspension was homogenized twice in a cream homogenizer and spun for five minutes at 500 g. in an M.S.E.

high speed 18 centrifuge at 4°C. The resulting supernatant was used either for microinjection or for preparing other cell fractions.

(b) 5000 g. pellet

This type of preparation will be referred to as a mitochondrial preparation as it contains at least 80% mitochondrial and is the cell fraction most frequently used in this work. The 500 g. supernatant was spun at 5,000 g. for 2 minutes at speed and then the supernatant was discarded. On some occasions this supernatant was saved for microinjection in control experiments.

The walls of the tube were wiped with absorbent tissue to remove lipids adhering to them. The top section of the pellet, containing a higher proportion of cilia and bacteria (Preer and Preer, 1959) was washed off with homogenization buffer. The centre section of the 5000 g. pellet was carefully resuspended in 0.1 ml. of homogenization buffer and placed in a serum tube in ice. The lower quarter of the pellet, containing a higher proportion of trichocysts was discarded.

(c) Preparation from a D_2O/H_2O Interface

This procedure was devised to obtain preparations of mitochondria purer than previous methods, without the excessively long times required (3 hours) by linear sucrose gradient techniques. The 500 g. supernatant was placed on a 20 ml. 50% aqueous sucrose cushion, itself supported on a 4 ml. 50% deuterium oxide-sucrose cushion in a 40 ml. tube. The D_2O sucrose cushion was inserted into the bottom of the tube just before use to prevent mixing of the D_2O and H_2O layers. This two step

gradient was then centrifuged at 10,000 g. for 15 minutes in a 3 x 40 swing-out rotor and allowed to come to rest without braking. The base of the tube was pierced and the H_2O/D_2O sucrose interfaces, containing a large proportion of the mitochondria, collected. This preparation was then diluted once with raf inose homogenization buffer so that the cells injected were not killed by the high sucrose concentrations.

7. Treatment of Mitochondrial Preparations with DNAse, RNAse and Detergent.

(a) DNAse and RNAse Treatment

A mitochondrial preparation was made as previously described except that the 500 g. supernatant was divided into two equivalent parts so as to produce two equivalent 5,000 g. pellets. One of these pellets was resuspended in a solution containing 0.215 M raf inose, 1.5 mg./ml. bovine serum albumin, 0.01 M potassium phosphate pH 6.2 and 1 mg./ml. of nuclease. The other pellet was resuspended in a similar buffer without nuclease.

Two nucleases were used in this study:

(i) DNAse II purified from hog spleen. This was used in preference to DNAse I as it has little or no magnesium requirement. Magnesium in concentrations as low as 10^{-4} molar causes extensive clumping of mitochondria and consequently renders the preparation impossible to inject.

(ii) Ribonuclease A purified from bovine pancreas and crystallized five time. This preparation is described as protease free.

(b) Detergent Treatment of Mitochondrial Preparations

The equivalent 5600 g. pellets were prepared as described previously; one of these was resuspended in 0.1 ml. raf inose homogenization buffer containing 1.5% Triton x 100 and the other was resuspended in 0.1 ml. of homogenization buffer without detergent.

Treated and untreated preparations were injected alternately with sensitive paramecia so as to eliminate errors due to differences in time between homogenization of the cells and injection into a cell.

(c) Assay for DNAse and RNAse activities in the Homogenisation Buffer

A solution was made up containing 1 mg/ml specific nuclease, 1.5 µg./ml. bovine serum albumin, 0.215 M rafinose and 0.001 m. potassium phosphate and preincubated at 0°C in an ice bath. The experiment was initiated by the addition of 0.5 ml. of a solution containing DNA or ^{14}C the nuclease solution. This resulted in a reaction mixture containing initially at least 500 T.C.A. precipitable counts per minute.

At various times 0.1 ml. aliquots were diluted with 2 ml. of 10% T.C.A., 0.1 M NaCl at 0°C. This was then filtered onto fibre glass disks and the T.C.A. precipitable radioactivity estimated by counting for ten minutes in a Nuclear Chicago scintillation counter.

8. Electron Microscopy

(a) Mitochondrial Pellets.

Mitochondrial pellets prepared as described in section 6b were resuspended in an Osminum fixative and spun at 15,000 g. for 30 minutes at 0°C. The fixative was prepared to the following formula (Jurand and Selman, 1968).

Solution A.	Sodium Veronal	14.714 gm.
	Sodium Acetate	9.714 gm.
	Distilled water to	500 ml.

Solution A is made up and kept at room temperature. Solution B is prepared freshly for each fixation.

was complete the cells were transferred to 50/50 araldite/ethanol in a solid watch glass. After 30 minutes the cells were transferred to pure araldite and stirred on a rotary shaker for 2 hours. They were then transferred individually to the top of a gelatin capsule containing partially prepolymerised araldite and polymerised at 60°C for 24 hours. After careful trimming, the single cells were sectioned on a Porter-Blum ultra microtome and mounted on colloidon carbon-coated grids.

Staining was carried out by floatation on a 1% solution of potassium permanganate containing 2.5% uranyl acetate for 20 minutes. Excess stain was removed by floating the grids on distilled water, then on 0.25% citric acid for 30 seconds, followed by three changes of distilled water. The sections were then examined in an A.E.I. 6B electron microscope at 60 kilovolts.

9. Control Experiments for Inter-syngen Transfers

Towards the end of each experiment, at least 10 sensitive cells of the same syngen as those used for the mitochondrial preparation were injected. The subsequent development of erythromycin resistance by these cells served to confirm that the mitochondrial preparation was still 'viable'.

In addition, at least 24 uninjected cells of each syngen were isolated into the medium used to select for erythromycin resistance to check that growth in this medium was specifically related to the acquisition of erythromycin resistance.

10. Starch Gel Electrophoresis

The method used has been described in detail by Tait (1969) and is essentially similar to that described by Smithies (1955) and Smith (1968).

250 ml. of cells is harvested on an oil testing centrifuge and the pelleted cells homogenized in a sucrose phosphate buffer. Mitochondria were prepared by differential centrifugation and homogenized in Triton X 100. A 75,000 g. supernatant was absorbed onto small pieces of chromatography paper which was then inserted into slits in a 9-11% starch gel (Connaught Research Laboratories). Electrophoresis was carried out at constant voltage at 0°C for 3.5 hours. The gels were specifically stained for fumerase activity using the dye MTT tetrazolium and then washed overnight in tap water.

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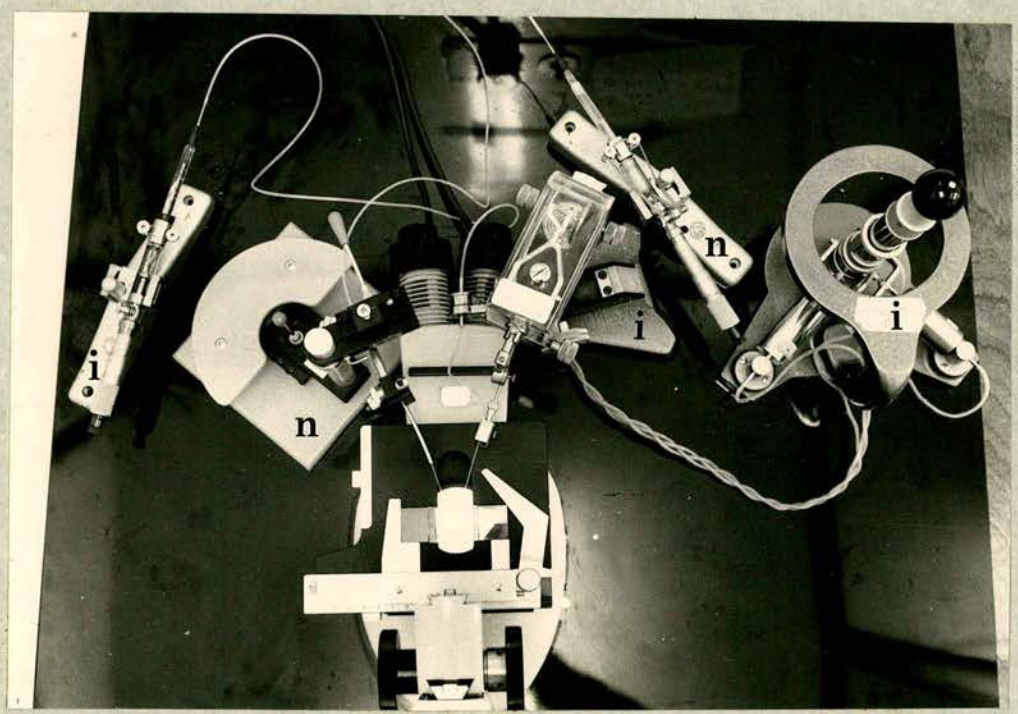

Figure 1: The apparatus used for microinjection. This shows the injection system (i), and the immobilization system (n).

Section II : The Technique of Microinjection

The aim of this technique is to insert a quantity of fluid into the cytoplasm of a single Paramecium cell.

1. Apparatus

The apparatus described here is similar to that used by Koizumi and Preer (1966). However the system has been considerably improved during the course of this work, so that it is now possible to inject large numbers of cells (up to 80) in a relatively short time (3 hours).

The components of the system are as follows (see Figure 1).

(a) Microscope

A Carl Zeiss Mark III stereo-zoom microscope fitted with a x 2 objective and x 25 eyepieces, one of which carries a graticule, is used to observe the injection. A maximum magnification of x 200 allows accurate placing of the injection needle in the cell. A Gillett and Sibert stage with concentric controls is damped to the illumination base of the microscope. This stage holds a perspex moist chamber of dimensions 25 mm. x 20 mm. x 0.5 mm. covered by a 32 mm. x 44 mm. coverslip (Chance No.2 grade). The paramecium cells to be injected are placed in small hanging drops around a central drop of mitochondrial preparation on the underside of this coverslip.

(b) The Injection System

An Agla microinjection unit (Wellcome) attached to a solid base, containing a Hamilton 50 microlitre syringe is connected by

1 mm. internal diameter polyethylene tubing (L.K.B.) to a pyrex glass microinjection needle with a tip of internal diameter < 3 microns. The whole system is filled with oil and should be completely air free so as to provide precise control of the pressure in the injection needle. The injection needle is held and controlled by a de Fonbrune pneumatic micromanipulator.

(C) The Cell Immobilization System

To immobilise the cell just prior to injection, the liquid in which the cell is swimming is partially removed. The cell is then held stationary by surface tension and friction against the coverslip. The liquid is removed by a second glass needle with a very large tip diameter (200 microns) connected to a second Agla micrometer syringe unit containing a 2 ml. glass syringe. This system is filled with a Dryl's solution (Dryl, 1965) containing 2 mg./ml. bovine albumin. The immobilization needle is positioned by a micromanipulator (Research Instruments) on which the vertical and horizontal motions are separated.

(d) Location of the Apparatus

The whole microinjection apparatus is placed on a 1 metre x 0.66 metre concrete paving slab supported by 2 inch rubber bungs placed on a firm metal framework. This is enclosed in a small room having space for the operator and an additional service binocular microscope on a bench at right angles to the injection equipment. This room is situated in an 18°C constant temperature room in order to prevent the rupture of cells which may occur at higher temperatures.

2. Preparation and adjustment of the apparatus

(a) Making microinjection needles

Needles for microinjection were made from pyrex tubing using an automatic electrode puller. The last 5 mm. of each needle was bent to an angle of between 12 to 18 degrees from its main axis with a microflame. The tips of the injection needles were broken off against a solid surface under a microscope to give an internal tip diameter of 3.0 $\pm$ 0.1 microns.

(b) Changing the microinjection needle

The Luer-Lock fitting is removed from the 50 microlitre syringe and filled with oil. The new needle is inserted into the tube and this is then reclamped in the micromanipulator. The needle is then repositioned in the field of view and filled with oil by pressing the Luer fitting firmly onto the syringe. This action forces the oil up the injection needle so expelling the air.

(c) Filling the Immobilisation system

When not in use the immobilisation system is kept full of ethanol to ensure sterility. The ethanol is rinsed out with 2 changes of distilled water and the system filled with Dryl's solution containing 2 mg./ml. B.S.A.

(d) Treatment of Cells.

Prior to microinjection the cells to be injected were placed in Dryl's/B.S.A. solution in a depression slide. This is to protect the cells against excessive tension produced during immobilisation. After microinjection the surviving cells are returned to fresh bacterized medium and left overnight at 18^{o}C.

3. The method of Microinjection.

(a) Individual freely swimming paramecia are placed in small hanging drops on a coverslip (Chance, 22 mm. x 40 mm., No.2). In the centre of the coverslip is placed a small drop of the cell extract to be injected. The coverslip is then inverted and placed on the moist chamber.

(b) A standard quantity of the cell extract, usually about 6 nanolitres, is drawn up into the injection needle. This is achieved by moving the oil/extract meniscus up the needle a given distance as measured by the eye piece micrometers.

(c) The free liquid supporting a cell is removed with the immobilisation system, so that the cell is held stationary against the coverslip

(d) The zoom lens is changed to give x 200 magnification and the injection needle is inserted into the cell. The best position for this is between the posterior contractile vacuole and the macronucleus, but other locations are possible. When the needle is seen to have penetrated the cell, the cell extract is gently expelled from the needle. Frequently the fluid from the needle can be seen to displace the cytoplasm in the cell.

(e) The injection needle is rapidly removed and the cell released by flooding the slide with the immobilisation system.

(f) If the cells or cell extract leave particulate matter on the outside or inside of the injection needle this is removed by rinsing the needle with a solution of 2% Triton X 100 and then rinsing with distilled water.

4. Factors affecting the success of microinjection

(a) Composition of the fluid in which the injection is carried out.

Initially, the variation in the physiological state of the cells and the variable surface tension of the bacterised grass medium in which the cells were growing, produced very variable injection success. For this reason a more defined medium with reduced surface tension was developed. The best medium was found to be Dryl's B.S.A. (see Section 2c). The use of this solution considerably simplified the technique of microinjection such that whereas initially it was only possible to inject approximately 5 cells per hour, it is now possible to inject 30-40 cells per hour.

(b) The angle of the microinjection needle

The precise angle of the injection needle makes with the coverslip was found to be very important for the successful injection of cells. If this angle is too low the needle will not penetrate the cell. If the angle is too high, the needle is pushed through the cell and the fluid to be injected is expelled on the other side of the cell. The optimum angle has been found to be between 12 and 18^{o} from the horizontal, but this varies with the exact nature of the needle tip and the physiological condition of the cells being injected.

(c) The size and shape of the microinjection needle.

The larger the needle tip diameter the more difficult it is to insert the needle into the cell. If the needle is too small it will not allow the free passage of the larger cytoplasmic components such as mitochondria and trychocysts. The optimum size of needle for this study was found to be one having an internal diameter of 3 microns.

(d) Physiological condition of the cells used for microinjection

Cells used for microinjection should be in late log phase or early stationary phase. At this stage of growth the cells are at their largest and have a greater ability to recover from injection than cells taken from cultures in early log phase or late stationary phase.

5. Quantitative Estimates

(a) Estimation of mitochondrial concentration

An estimate of the numbers of mitochondria per unit volume in a preparation of mitochondria was obtained by counting a sample of the preparation which had been diluted tenfold in a special 1/400 mm^2 x 0.01 mm grid square counting chamber (Hawkesly).

For each preparation the number of mitochondria-like objects in 10 groups of ten squares on the grid were counted. This technique was found to give an estimate accurate to $\pm$ 30% ($\pm$ 2 standard deviations) of the concentration of mitochondria-like objects counted.

(b) Estimation of the volume inserted by injection

Paraffin oil was drawn into the injection needle a given distance, measured by the eyepiece micrometer. This was normally 20 micrometer units or 226 microns length. This oil was gently expelled into a solution containing detergent, and the diameter of the spherical drop so formed measured with the micrometer. Oil occupying a length of 226 microns in the needle produced a sphere of diameter 22.6 microns. The volume of this sphere was found to be 6,000 cubic microns or 6 nanolitres. No siginficant variation was found between neddles with the same internal tip diameter.

(c) Calculation of the dilution required to inject a known mean number of mitochondria per cell.

Let:- i) a be the initial undiluted concentration of mitochondria-like objects

ii) A be the number of mitochondria like objects seen per square

Then $a = \frac{A \times 10^2}{2.5 \times 10^4}$ per cubic micron

as the volume of a counting grid square is 2.5×10^4 ^{3}.

The volume of the injection, $v = 6 \times 10^3$ ^{3}

Therefore, the number of mitochondria injected if the solution is not diluted is $a.v. = 24A$.

The number of mitochondria injected per cell if the original preparation is diluted d-fold = $\frac{av}{d}$.

If we wish to inject y mitochondria-like objects per injection, then $y = \frac{a.v}{d}$.

Typical values are $A = 11.01$

$y = 5$ (or 1)

Then, $d = 53$

The original mitochondrial preparation is then diluted 1:53 so as to make a solution such that an average of five mitochondria are injected into each cell.

CHAPTER I

The Nature and Location of Cytoplasmically Inherited Erythromycin Resistance in Paramecium aurelia.

Introduction

Non-Mendelian mutants of Saccaromyces cerevisiae resistant to the antibacterial antibiotic erythromycin have been isolated by several workers (Clark-Walker and Linnane, 1966; Lamb, Clark-Walker and Linnane, 1967; Coen et al., 1970). Experiments involving reciprocal crosses between antibiotic resistant markers and the ρ factor responsible for petite forms in yeast have shown that erythromycin resistance is caused by a heritable change in mitochondrial DNA (Thomas and Wilkie, 1968; Linnane et al., 1968a).

In Paramecium, Beale (1969) was able to obtain mutant cells having the ability to grow normally in the presence of 250 μg/ml erythromycin-sensitive, wild-type cells are unable to grow for more than a few fissions in concentrations of erythromycin as low as 70 μg/ml. This erythromycin resistance was shown to be due to a cytoplasmic factor. Adoutte and Beisson (1971 and 1972) and Beale et al., 1972 isolated many mutants of P.aurelia, resistant to both erythromycin and chloramphenicol, all of which seem to be determined by cytoplasmic factors.

The aim of this work was to locate the site of cytoplasmically inherited erythromycin resistance in Paramecium aurelia. Using the technique of microinjection, evidence has been obtained that resistance to erythromycin is located in the mitochondria, probably on the mitochondrial DNA. Preliminary results are discussed which suggest that only one mitochondria is required to transfer erythromycin resistance into sensitive cells.

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Mitochondrial Genetics in *Paramecium*

RESISTANCE to erythromycin and chloramphenicol in yeast has been shown to be controlled by non-Mendelian genetic factors in mitochondrial DNA[1,2]. Because paramecium has particular advantages for the study of cytoplasmic genetics[3], we have made some genetic and biochemical studies on drug-resistant variants of this ciliate, working with syngens 1 and 4 of *P. aurelia*.

When paramecia are placed in bacterized lettuce medium (*p*H 6.8–7.0) containing 0.25 mg ml.$^{-1}$ erythromycin, growth stops after one to three fissions, but thereafter the ciliates remain alive for a considerable time. Between two and four weeks afterwards, erythromycin-resistant mutants appear[4,5], at a frequency of about 1 per 1,000 paramecia initially present. Paramecia resistant to chloramphenicol (0.25 mg ml.$^{-1}$) have also been obtained, but only after treatment with a mutagenic chemical (nitrosoguanidine). The variants have been shown to be stable for between one and two years when grown in media lacking antibiotics.

Genetic tests have shown, both in syngens 1 and 4, that resistance to both drugs is inherited through the cytoplasm at conjugation. When there is no cytoplasmic mixing at conjugation between sensitive and resistant paramecia (the normal situation), one of the ex-conjugant clones consists of resistant animals and the other sensitive. When there is cytoplasmic mixing, which occurs commonly in stock 51 (syngen 4), but rarely in other stocks unless induced by special treatments[6], both ex-conjugants produce drug-resistant animals. The cytoplasmic basis of inheritance of erythromycin resistance was confirmed by a programme of repeated back-crossing to sensitive animals. No effect of nuclear genes on drug resistance has been shown, but the matter has not been intensively studied.

Cytoplasmic mixing between erythromycin-resistant and chloramphenicol-resistant conjugants results in the production of paramecia capable of growing in either antibiotic, but not in medium containing both. Such "mixed" clones, after growth in one antibiotic for a few (ten to fifteen) fissions, lose their resistance to the other antibiotic. When grown in drug-free medium for a hundred or more fissions, however, "mixed" clones may retain their capacity to grow in the presence of either antibiotic, although lines containing animals resistant to erythromycin alone appear sporadically. Thus in one experiment, six hybrid clones containing mixtures of cytoplasm from stocks 51-E_I^R (EDIN) (erythromycin-resistant) and 51-C_I^R (EDIN) (chloramphenicol-resistant) were grown in normal medium. Chloramphenicol resistance was lost after forty-seven fissions in one clone, after 106 fissions in three clones and after 195 fissions in the sixth clone. Erythromycin resistance was not lost at all in this experiment.

Similar experiments were done with clones containing mixtures of resistant and sensitive cytoplasm and the results indicated that there was a slight selective advantage of "wild type" or sensitive (51-E^SC^S) cytoplasm over 51-E_I^R and of 51-E_I^R over 51-C_I^R, but these are probably strain or mutant-specific characteristics, judging by the different results obtained by Adoutte and Beisson[7].

Attempts to obtain "recombination" between 51-E_I^R (EDIN) and 51-C_I^R (EDIN) cytoplasmic components by selection of doubly resistant animals have so far failed, although such doubly resistant types have been obtained by mutation.

In an attempt to identify the particular cytoplasmic factor associated with the transfer of antibiotic resistance, various cell fractions were prepared from erythromycin-resistant paramecia (168-E_I^R (EDIN) and injected into sensitive paramecia (168-E^S (EDIN)) using a micro-injection technique[8].

Two methods of preparing cell fractions for micro-injection

Fig. 1 Electron micrograph of an osmium fixed mitochondrial preparation, pelleted and embedded in 'Araldite'. M, Mitochondria; b, bacteria; s, fragments of surface membrane; c, cilia; t, trichocysts.

into sensitive cells were used. (i) Packed cells were resuspended in four volumes of a sucrose buffer (0.3 M sucrose, 100 mg ml.$^{-1}$ bovine serum albumin, 0.001 M potassium phosphate, *p*H = 7.0) at 4° C and were twice homogenized in a cream homogenizer. After centrifugation at 1,500*g* for 7 min, the supernatant, which would be expected to contain most cell components except macronuclei and large pieces of pellicle, was used for micro-injection. Of a total of thirty-four surviving cells injected with this type of preparation, twenty-three developed the ability to grow in erythromycin after a delay of 9–15 days at 24° C. None of the seventy-four uninjected controls became resistant. (ii) Mitochondria were prepared by differential centrifugation in raffinose buffer[9]. The centre section of the "mitochondrial" pellet was resuspended in the smallest possible volume (about 0.5 ml.) of the raffinose homogenization buffer and injected into paramecia as soon as possible.

An estimate of the composition of this preparation was obtained by examination of an osmium-fixed pellet under the electron microscope (Fig. 1). The following percentages of various particulate components were found: mitochondria, 80%; bacteria, 10%; fragments of surface membrane, 4%; cilia, 1%; trichocysts, 4%; other, 1%. These percentages

represent the numbers of each component in relation to the total number of particles counted.

Of sixty-four animals injected with this preparation, fifty-four became resistant after 4–15 days while none of the 132 uninjected controls developed resistance. Five cells injected with the post-mitochondrial supernatant and eleven cells injected with preparations from sensitive cells did not become resistant. The spontaneous mutation rate is of the order one in 10^3 cells (one in 10^7 mitochondria), so these results are felt to be highly significant. It is also important to point out that positive results were only obtained following the use of an injection needle with an internal diameter of not less than 3.0 μm and that this corresponds well with the size of the mitochondria of paramecium (2–4 μm diameter). Although the injected material did not consist exclusively of mitochondria, our results are entirely consistent with the view that transfer of resistance from one cell to another was in fact mediated by these organelles.

Our further work has concerned the site and biochemical nature of the alterations produced in drug-resistant paramecia. Erythromycin and chloramphenicol are both known to inhibit protein synthesis on bacterial ribosomes, and alterations in the

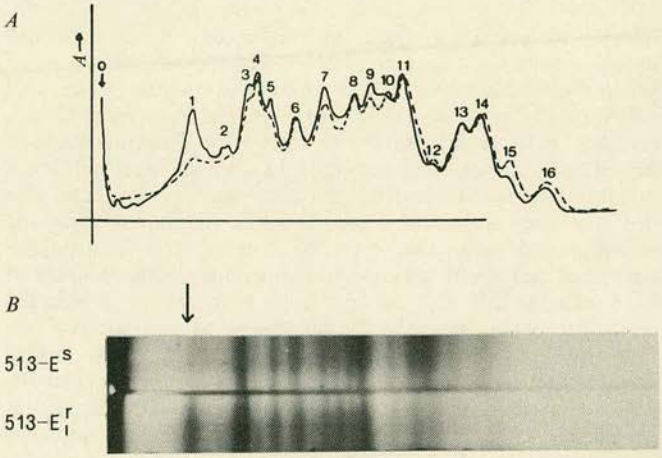

Fig. 2 *a*, Comparison of densitometer traces of stained acrylamide gels of separated "crude" mitochondrial ribosomal proteins from strains 513-E^S (EDIN) (- - -) and 513-E_1^R (EDIN) (——). The proteins are numbered 1–16 from the origin, marked ○. *b*, Photograph of the acrylamide gels scanned in *a*. The difference between the two strains in band 1 is indicated by the arrow.

ribosomal proteins have been shown to occur in erythromycin-resistant strains[10], so we have investigated the possibility of similar alterations in the mitochondrial ribosomal proteins of drug-resistant paramecia.

Twice-washed mitochondria were prepared by differential centrifugation[9] and then lysed by homogenization in 2% 'Triton X-100 TMK' (50 mM Tris-HCl, *p*H 7.5, 10 M $MgCl_2$, 10 mM KCl), using a 'Teflon' glass homogenizer. The mitochondrial ribosomes were separated by differential centrifugation of this lysate[11] and the proteins extracted from the crude ribosomal pellet by treatment with 4 M LiCl–8 M urea at 4° C for 12 h, the RNA being removed by centrifugation after this treatment[12]. The ribosomal proteins from resistant and sensitive strains were then compared by acrylamide electrophoresis at *p*H 4.5[12,13]. Electrophoresis was carried out for 4 h at 4 mA gel^{-1} at 4° C. The gels were stained with 1% naphthalene black for 1 h, destained electrolytically in 7% acetic acid and scanned using a Joyce-Loebl ultraviolet scanner.

The electrophoretic pattern of these proteins shows some sixteen bands (Fig. 2) and differences have been observed between sensitive and drug resistant strains. For example, comparison of 513-E^S (EDIN) (syngen 1, sensitive) with 513-E_1^R (EDIN) (syngen 1, spontaneously erythromycin-resistant) shows a quantitative alteration in a single protein band (No. 1) (Fig. 2). The difference observed was found consistently in different electrophoretic runs and using independent preparations of the crude ribosomal proteins from the two strains. The ratio of the area under peaks 3 and 4 to the area under peak 1 has been calculated for the two strains and the difference between this ratio for 513-E^S (EDIN) and 513-E_1^R (EDIN) was shown to be significant. While it is realized that the preparation of mitochondrial ribosomal proteins is crude, these results are consistent with the view that the change from sensitivity to erythromycin resistance involves the alteration of one or more proteins associated with the mitochondrial ribosome.

The precise nature of this difference has not been ascertained but various possible explanations are now being investigated. Attempts are also being made to establish unequivocally the mitochondrial ribosomal origin of this protein.

Our results show that erythromycin resistance and chloramphenicol resistance in *P. aurelia* are due to cytoplasmic genetic factors located in the mitochondria (confirming the findings with yeast). These mitochondrial "genes" modify certain proteins associated with the mitochondrial ribosomes. The special features of the paramecium material, such as the very large size of the "cells", permitting transfer of mitochondria by micro-injection, the fact that the reaction of individual

cells to the drugs can be studied, and the large size and number of mitochondria per cell (as compared with yeast) will make possible the elucidation of new features of mitochondrial genetics. Further technical and other details of this work will be published elsewhere.

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A New Method for Studying the Genetic Control of Specific Mitochondrial Proteins in *Paramecium aurelia*

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Summary. Mitochondria from one syngen (or sub-species) of *Paramecium aurelia* have been introduced into a different syngen by preparing erythromycin-resistant mitochondria from syngen 1 and micro-injecting them into erythromycin-sensitive syngen 7 cells. The recipient sensitive cells were then placed in erythromycin to inhibit the replication of the sensitive mitochondria. Such selected clones contain a syngen 7 nucleus but a mitochondrial genome which is derived from syngen 1 erythromycin-resistant mitochondria.

Using this method it has been shown that the mitochondrial enzyme fumarase is not coded by the mitochondrial genome, and by implication, is coded by the nuclear genome. The use of this technique as a method for determining if specific mitochondrial proteins are controlled by nuclear or mitochondrial genes is discussed.

Introduction

It is now well established that mitochondria contain DNA (Borst and Kroon, 1969; Ashwell and Work, 1970; Borst, 1972), but many of the specific functions of this DNA remain to be determined. Hybridization studies with mitochondrial ribosomal RNA and transfer RNA have shown that mitochondrial DNA codes for these molecules (Borst and Aaij, 1969; Nass and Buck, 1970; Attardi *et al.*, 1970; Borst and Grivell, 1971; Aloni and Attardi, 1971a, b and c). In animal mitochondria the RNA-DNA hybridization plateaus indicate that 20–25% of the total genome codes for the mitochondrial ribosomal and transfer RNA (Borst, 1972). It seems likely that the remaining DNA sequences code for certain mitochondrial proteins and several experimental approaches are available for investigating which mitochondrial proteins are involved. These approaches can be summarised as follows (Borst, 1972):

(i) amino acid incorporation into mitochondrial proteins in the presence of inhibitors of mitochondrial or cytoplasmic protein synthesis, followed by identification of the labelled products;

(ii) analysis of the proteins labelled when isolated mitochondria are incubated with labelled amino acids;

(iii) identification of mitochondrial proteins synthesised in the absence of mitochondrial DNA in petite mutants with no m-DNA;

(iv) identification of altered proteins in drug resistant, cytoplasmically inherited mutants.

Evidence supplied by these approaches is often difficult to interpret unambiguously (Nass, 1969; Ashwell and Work, 1970; Rabinowitz and Swift, 1970; Kroon and de Vries, 1970; Borst and Grivell, 1971; Mahler *et al.*, 1971). In the case of approaches (i) and (ii) the synthesis of certain proteins may depend on

components from both nuclear and mitochondrial genomes, so that one may underestimate the number of proteins coded for by the nucleus or mitochondria. Also, mitochondrial messenger RNA may be translated on cytoplasmic ribosomes or nuclear messenger RNA may be translated on mitochondrial ribosomes and this would confuse the interpretation of these results. Using petite cells, described in (iii), the mitochondria are grossly altered and some of the proteins coded for by the nucleus may depend on a complete mitochondrial structure for incorporation and synthesis. The major disadvantage of approach (iv) is the limited number of drugs and resistant mutants which are available; this approach also requires that crosses can be made between resistant and sensitive cells.

The approach described in this paper does not depend on the use of extensively altered mitochondria or drug inhibited systems. Cytoplasmically inherited erythromycin-resistant mutants have been obtained in *Paramecium* (Beale, 1969; Adoutte and Beisson, 1970) and evidence has been obtained, showing that the mutation is mitochondrial (Beale *et al.*, 1972). In the present study, the original sensitive mitochondrial population of a cell has been replaced by an erythromycin-resistant population of mitochondria, derived from a sub-species which is sexually isolated from the recipient cell. This approach allows the study of clones which derive their mitochondrial genome from one syngen (or sub-species) and their nuclear genome from another. Intersyngenic variation of mitochondrial proteins allows the localisation of the genetic information for a variant protein to be made by micro-injection of mitochondria between sexually isolated sub-species. Fumarase has been shown to differ in electrophoretic mobility between syngens (Tait, 1970a) and in this paper, this difference is used to show that fumarase is controlled by the nuclear genome.

Materials and Methods

Stocks: stocks 168 (syngen 1), 227 (syngen 7) and the spontaneous erythromycin resistant mutant 168 E_1^R (Edin), derived from 168, of *Paramecium aurelia* were used. The mutant stock grows at normal rate in 250 μg/ml of erythromycin.

Cultures: stocks were cultured on a grass infusion, buffered with phosphate, and bacterized with *Aerobacter aerogenes* (Sonneborn, 1950) at 25° C.

Mitochondria: 10 L. cultures of Paramecium were filtered through cotton wool and the cells pelleted by continuous flow centrifugation at a flow rate of 700 ml/min at 500 g. The packed cells were resuspended in Dryl's solution and pelleted by centrifugation at 1500 r.p.m. for 4 min. in an M.S.E. oil-testing centrifuge. The pelleted cells were disrupted in 10 volumes of buffered raffinose (Preer 1959) by homogenisation in a cream homogeniser. Mitochondria were then prepared by differential centrifugation as previously described (Beale *et al.*, 1972).

Micro-injection: The technique used was essentially that of Koisumi and Preer (1966) but with modifications to be described elsewhere (Knowles, in press). A thick suspension of mitochondria prepared from resistant syngen 1 cells was micro-injected into single sensitive syngen 7 cells. The viability of the mitochondrial preparation was estimated by injecting it into sensitive syngen 1 cells. This was done because intra syngen micro-injections of mitochondria are usually much more successful than intersyngen micro-injections.

Drug selection: After micro-injection, cells were transferred to bacterized medium and after one fission at 18° C (in the absence of erythromycin) were transferred singly to medium containing 250 μg/ml erythromycin and incubated at 25° C.

Extracts for electrophoresis: 250 ml cultures of stocks were concentrated by centrifugation and the pelleted cells homogenised as previously described (Tait, 1970a).

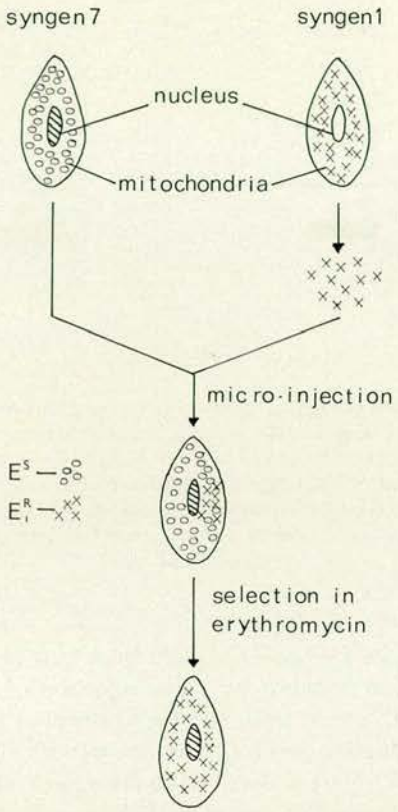

Fig. 1. Transfer of mitochondria by micro-injection between different syngens, showing the origin of the syngen 1/7 "hybrid"

Results

Syngen 7 sensitive cells were micro-injected with mitochondria prepared from syngen 1 resistant cells and after 3 weeks of incubation at 25° C in erythromycin-containing medium, 2 out of a total of 40 microinjected syngen 7 sensitive cells became resistant. None of the 96, un-injected syngen 7 sensitive controls became resistant. It should be noted that the transfer of resistance by micro-injection between resistant and sensitive stocks of the same syngen is usually 70–100%. (Beale *et al.*, 1972). Thus the transfer of resistance implies, firstly, that the mitochondrial genome of syngen 1 is able to be incorporated into a cell containing a syngen 7 nucleus as shown in Fig. 1. Secondly, the considerable drop in the percentage intersyngen resistance transfer as compared to intrasyngen suggests that there are differences between the mitochondria of the two syngens.

The use of this technique for determining the nuclear or mitochondrial control of specific mitochondrial proteins requires the presence of intersyngen mitochondrial protein differences. It is known that syngens 1 and 7 differ as regards the electrophoretic mobility of fumarase (Tait, 1970a) and so the mitochondrial or nuclear location of the genetic control of this enzyme can be determined using

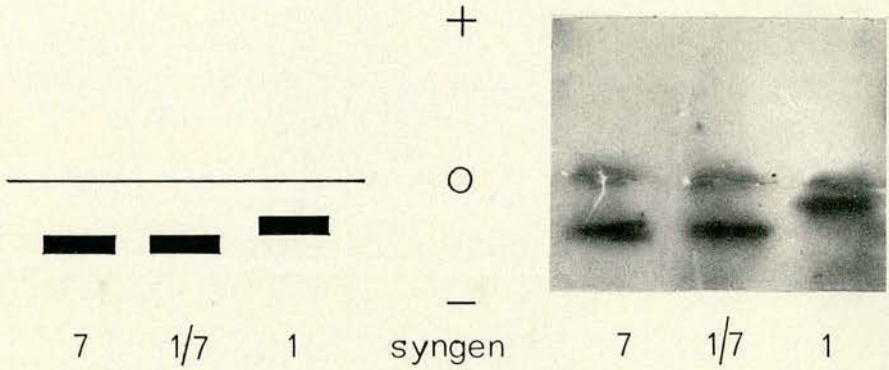

Fig. 2. Starch gel zymogram of fumarase, showing electrophoresis of extracts from syngen 1 ($168E_1^R$), syngen 7 (227) and syngen 7 cells micro-injected with syngen 1 erythromycin resistant mitochondria (7/1). Electrophoresis was carried out for 3 hours at 0° C in a 0.01 M phosphate-citrate buffer pH=5.5. Enzyme activity was localised by overlaying the gel with 30 mls of staining solution (3.9 mg/ml sodium fumarate, 0.25 mg/ml NAD, 0.2 mg/ml MTT-tetrazolium, 0.02 mg/ml PMS and 1 i.u. malic dehydrogenase in 0.1 M phosphate buffer pH=7.4) and incubating at 35° C

the micro-injected syngen 7 clones. Extracts from cells of stocks $168E_1^R$, 227 and 7/1 (syngen 7 cells made resistant by micro-injection of erythromycin resistant mitochondria from $168E_1^R$) were made and then subjected to starch gel electrophoresis, followed by staining the gels for fumarase activity. As can be seen in Fig. 2, the "hybrid 7/1" stock shows a band of fumarase activity identical in mobility to that of syngen 7. This result establishes that fumarase is not controlled by the mitochondrial genome and by inference must be controlled by the nuclear genome.

Conclusion—Discussion

Certain soluble mitochondrial enzymes have been shown to be controlled by nuclear genes in both *Paramecium* (Tait, 1968; Tait, 1970b) and other organisms (Munkres *et al.*, 1965; Munkres and Woodward, 1967; Davidson and Cortner, 1967; Longo and Scandalios, 1969; Davidson *et al.*, 1970) using within-species genetic analysis. The result presented in this paper provides further evidence for the idea that most or possibly all the soluble mitochondrial enzymes are controlled by the nuclear genome, but uses a new method for establishing the localisation of the genetic control.

The more general application of this technique of micro-injection for determining the control of specific mitochondrial proteins will depend on the elucidation of further intersyngen differences in mitochondrial proteins; fumarase is the only mitochondrial protein so far shown to have different forms in syngen 1 and syngen 7 cells. However, as only 5 enzymes (glutamate dehydrogenase, succinate dehydrogenase, β-hydroxybutyrate dehydrogenase, iso-citrate dehydrogenase and fumarase) have been compared so far, it is reasonable to expect that further differences will be observed when more proteins are examined. This type of study is being undertaken at the present time.

The aquisition of erythromycin-resistance by the micro-injected syngen 7 sensitive cells could occur by a number of mechanisms which must be considered before we can definitely state that syngen 1 mitochondria are able to replicate in a syngen 7 cell. Three possible mechanisms can be considered:

(i) the syngen 7 resistant clones arise by spontaneous mutation;

(ii) the syngen 7 mitochondria aquire the resistance by "recombination" with the micro-injected syngen 1 erythromycin resistant mitochondria, by a mechanism involving the incorporation of an erythromycin-resistant "gene" from syngen 1.

(iii) the syngen 7 sensitive mitochondria are eliminated by erythromycin and the syngen 1 resistant mitochondria replicate to produce mitochondria with their nuclear component from syngen 7 and their mitochondrial component from syngen 1.

The possibility that the syngen 7 cells aquire resistance by spontaneous mutation (i) can be eliminated since it is known that the spontaneous mutation rate is of the order of 1 per 1000 cells and this is confirmed by the absence of erythromycin-resistant cells amongst the un-injected controls, compared to the higher frequency of aquisition of resistance following micro-injection (1 in 20 cells).

The reduction in frequency of transfer of erythromycin resistance from 70–100% in intrasyngen experiments to about 5% in intersyngen experiments might be explained by a requirement for recombination in intersyngen transfers. Two pieces of evidence make recombination an unlikely possibility. Repeated attempts to obtain recombination between erythromycin and chloramphenicol resistant markers in syngen 4 (Beale *et al.*, 1972; Adoutte and Beisson, 1972) have been made but no recombination has been obtained. This is in marked contrast to results obtained with yeast (Thomas and Wilkie, 1968; Coen *et al.*, 1970; Wilkie, 1970) where recombination between similar markers can be obtained readily. The negative results with *Paramecium* suggest that mitochondrial recombination between these two markers occurs extremely rarely, if at all, in *Paramecium* and suggests that recombination may be a rare event in general.

Preliminary results with resistance transfer between syngens 1 and 5 shows a similar lowered percentage of transfer of resistance when syngen 1 resistant mitochondria are micro-injected into syngen 5 sensitive cells. When resistant mitochondria, prepared from the "hybrid" syngen 1/5 resistant cells are micro-injected into syngen 1 sensitive cells, 70–100% transfer of resistance is observed. This implies that the syngen 1 resistant mitochondrial genome is unchanged after replicating within the syngen 5 cell. If resistant mitochondria are prepared from the syngen 1 cells which have been previously micro-injected with mitochondria from the "hybrid" syngen 1/5 cells and then micro-injected back into syngen 5 sensitive cells, the same lowered percentage transfer of resistance is observed as that observed initially. If recombination between the mitochondrial genomes of syngens 1 and 5 had occurred one would expect an increased percentage resistance transfer back into syngen 5. Further work is in progress to establish these findings more definitely.

Thus, the most likely explanation for the results described here is that the whole syngen 1 mitochondrial genome is transferred into syngen 7 cells. The reduced frequency of transfer is possibly due to the interaction of the syngen 7

nuclear genome and the syngen 1 mitochondrial genome. It is hoped that by extending the search for mitochondrial protein differences between syngens, this approach will provide a valuable contribution to the elucidation of the informational content of mitochondrial DNA.

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Summary. Mitochondria from one syngen (or sub-species) of *Paramecium aurelia* have been introduced into a different syngen by preparing erythromycin-resistant mitochondria from syngen 1 and micro-injecting them into erythromycin-sensitive syngen 7 cells. The recipient sensitive cells were then placed in erythromycin to inhibit the replication of the sensitive mitochondria. Such selected clones contain a syngen 7 nucleus but a mitochondrial genome which is derived from syngen 1 erythromycin-resistant mitochondria.

Using this method it has been shown that the mitochondrial enzyme fumarase is not coded by the mitochondrial genome, and by implication, is coded by the nuclear genome. The use of this technique as a method for determining if specific mitochondrial proteins are controlled by nuclear or mitochondrial genes is discussed.

Introduction

It is now well established that mitochondria contain DNA (Borst and Kroon, 1969; Ashwell and Work, 1970; Borst, 1972), but many of the specific functions of this DNA remain to be determined. Hybridization studies with mitochondrial ribosomal RNA and transfer RNA have shown that mitochondrial DNA codes for these molecules (Borst and Aaij, 1969; Nass and Buck, 1970; Attardi *et al.*, 1970; Borst and Grivell, 1971; Aloni and Attardi, 1971a, b and c). In animal mitochondria the RNA-DNA hybridization plateaus indicate that 20–25% of the total genome codes for the mitochondrial ribosomal and transfer RNA (Borst, 1972). It seems likely that the remaining DNA sequences code for certain mitochondrial proteins and several experimental approaches are available for investigating which mitochondrial proteins are involved. These approaches can be summarised as follows (Borst, 1972):

(i) amino acid incorporation into mitochondrial proteins in the presence of inhibitors of mitochondrial or cytoplasmic protein synthesis, followed by identification of the labelled products;

(ii) analysis of the proteins labelled when isolated mitochondria are incubated with labelled amino acids;

(iii) identification of mitochondrial proteins synthesised in the absence of mitochondrial DNA in petite mutants with no m-DNA;

(iv) identification of altered proteins in drug resistant, cytoplasmically inherited mutants.

Evidence supplied by these approaches is often difficult to interpret unambiguously (Nass, 1969; Ashwell and Work, 1970; Rabinowitz and Swift, 1970; Kroon and de Vries, 1970; Borst and Grivell, 1971; Mahler *et al.*, 1971). In the case of approaches (i) and (ii) the synthesis of certain proteins may depend on

components from both nuclear and mitochondrial genomes, so that one may underestimate the number of proteins coded for by the nucleus or mitochondria. Also, mitochondrial messenger RNA may be translated on cytoplasmic ribosomes or nuclear messenger RNA may be translated on mitochondrial ribosomes and this would confuse the interpretation of these results. Using petite cells, described in (iii), the mitochondria are grossly altered and some of the proteins coded for by the nucleus may depend on a complete mitochondrial structure for incorporation and synthesis. The major disadvantage of approach (iv) is the limited number of drugs and resistant mutants which are available; this approach also requires that crosses can be made between resistant and sensitive cells.

The approach described in this paper does not depend on the use of extensively altered mitochondria or drug inhibited systems. Cytoplasmically inherited erythromycin-resistant mutants have been obtained in *Paramecium* (Beale, 1969; Adoutte and Beisson, 1970) and evidence has been obtained, showing that the mutation is mitochondrial (Beale *et al.*, 1972). In the present study, the original sensitive mitochondrial population of a cell has been replaced by an erythromycin-resistant population of mitochondria, derived from a sub-species which is sexually isolated from the recipient cell. This approach allows the study of clones which derive their mitochondrial genome from one syngen (or sub-species) and their nuclear genome from another. Intersyngenic variation of mitochondrial proteins allows the localisation of the genetic information for a variant protein to be made by micro-injection of mitochondria between sexually isolated sub-species. Fumarase has been shown to differ in electrophoretic mobility between syngens (Tait, 1970a) and in this paper, this difference is used to show that fumarase is controlled by the nuclear genome.

Materials and Methods

Stocks: stocks 168 (syngen 1), 227 (syngen 7) and the spontaneous erythromycin resistant mutant 168 E_1^R (Edin), derived from 168, of *Paramecium aurelia* were used. The mutant stock grows at normal rate in 250 μg/ml of erythromycin.

Cultures: stocks were cultured on a grass infusion, buffered with phosphate, and bacterized with *Aerobacter aerogenes* (Sonneborn, 1950) at 25° C.

Mitochondria: 10 L. cultures of Paramecium were filtered through cotton wool and the cells pelleted by continuous flow centrifugation at a flow rate of 700 ml/min at 500 g. The packed cells were resuspended in Dryl's solution and pelleted by centrifugation at 1500 r.p.m. for 4 min. in an M.S.E. oil-testing centrifuge. The pelleted cells were disrupted in 10 volumes of buffered raffinose (Preer 1959) by homogenisation in a cream homogeniser. Mitochondria were then prepared by differential centrifugation as previously described (Beale *et al.*, 1972).

Micro-injection: The technique used was essentially that of Koisumi and Preer (1966) but with modifications to be described elsewhere (Knowles, in press). A thick suspension of mitochondria prepared from resistant syngen 1 cells was micro-injected into single sensitive syngen 7 cells. The viability of the mitochondrial preparation was estimated by injecting it into sensitive syngen 1 cells. This was done because intra syngen micro-injections of mitochondria are usually much more successful than intersyngen micro-injections.

Drug selection: After micro-injection, cells were transferred to bacterized medium and after one fission at 18° C (in the absence of erythromycin) were transferred singly to medium containing 250 μg/ml erythromycin and incubated at 25° C.

Extracts for electrophoresis: 250 ml cultures of stocks were concentrated by centrifugation and the pelleted cells homogenised as previously described (Tait, 1970a).

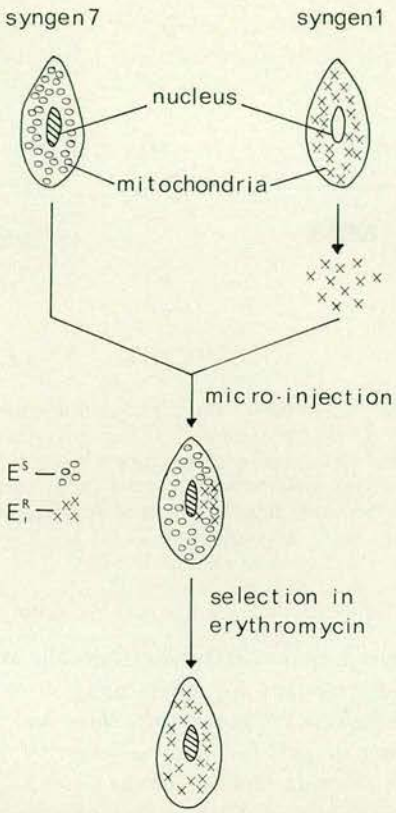

Fig. 1. Transfer of mitochondria by micro-injection between different syngens, showing the origin of the syngen 1/7 "hybrid"

Results

Syngen 7 sensitive cells were micro-injected with mitochondria prepared from syngen 1 resistant cells and after 3 weeks of incubation at 25° C in erythromycin-containing medium, 2 out of a total of 40 microinjected syngen 7 sensitive cells became resistant. None of the 96, un-injected syngen 7 sensitive controls became resistant. It should be noted that the transfer of resistance by micro-injection between resistant and sensitive stocks of the same syngen is usually 70–100%. (Beale *et al.*, 1972). Thus the transfer of resistance implies, firstly, that the mitochondrial genome of syngen 1 is able to be incorporated into a cell containing a syngen 7 nucleus as shown in Fig. 1. Secondly, the considerable drop in the percentage intersyngen resistance transfer as compared to intrasyngen suggests that there are differences between the mitochondria of the two syngens.

The use of this technique for determining the nuclear or mitochondrial control of specific mitochondrial proteins requires the presence of intersyngen mitochondrial protein differences. It is known that syngens 1 and 7 differ as regards the electrophoretic mobility of fumarase (Tait, 1970a) and so the mitochondrial or nuclear location of the genetic control of this enzyme can be determined using

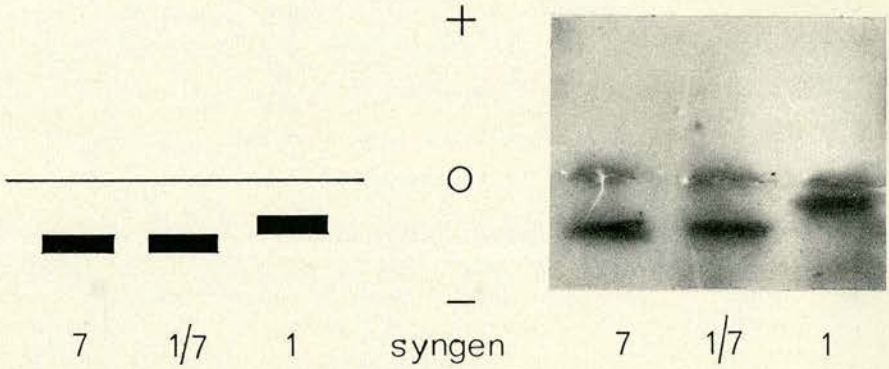

Fig. 2. Starch gel zymogram of fumarase, showing electrophoresis of extracts from syngen 1 ($168E_1^R$), syngen 7 (227) and syngen 7 cells micro-injected with syngen 1 erythromycin resistant mitochondria (7/1). Electrophoresis was carried out for 3 hours at 0° C in a 0.01 M phosphate-citrate buffer pH=5.5. Enzyme activity was localised by overlaying the gel with 30 mls of staining solution (3.9 mg/ml sodium fumarate, 0.25 mg/ml NAD, 0.2 mg/ml MTT-tetrazolium, 0.02 mg/ml PMS and 1 i.u. malic dehydrogenase in 0.1 M phosphate buffer pH=7.4) and incubating at 35° C

the micro-injected syngen 7 clones. Extracts from cells of stocks $168E_1^R$, 227 and 7/1 (syngen 7 cells made resistant by micro-injection of erythromycin resistant mitochondria from $168E_1^R$) were made and then subjected to starch gel electrophoresis, followed by staining the gels for fumarase activity. As can be seen in Fig. 2, the "hybrid 7/1" stock shows a band of fumarase activity identical in mobility to that of syngen 7. This result establishes that fumarase is not controlled by the mitochondrial genome and by inference must be controlled by the nuclear genome.

Conclusion—Discussion

Certain soluble mitochondrial enzymes have been shown to be controlled by nuclear genes in both *Paramecium* (Tait, 1968; Tait, 1970b) and other organisms (Munkres *et al.*, 1965; Munkres and Woodward, 1967; Davidson and Cortner, 1967; Longo and Scandalios, 1969; Davidson *et al.*, 1970) using within-species genetic analysis. The result presented in this paper provides further evidence for the idea that most or possibly all the soluble mitochondrial enzymes are controlled by the nuclear genome, but uses a new method for establishing the localisation of the genetic control.

The more general application of this technique of micro-injection for determining the control of specific mitochondrial proteins will depend on the elucidation of further intersyngen differences in mitochondrial proteins; fumarase is the only mitochondrial protein so far shown to have different forms in syngen 1 and syngen 7 cells. However, as only 5 enzymes (glutamate dehydrogenase, succinate dehydrogenase, β-hydroxybutyrate dehydrogenase, iso-citrate dehydrogenase and fumarase) have been compared so far, it is reasonable to expect that further differences will be observed when more proteins are examined. This type of study is being undertaken at the present time.

The aquisition of erythromycin-resistance by the micro-injected syngen 7 sensitive cells could occur by a number of mechanisms which must be considered before we can definitely state that syngen 1 mitochondria are able to replicate in a syngen 7 cell. Three possible mechanisms can be considered:

(i) the syngen 7 resistant clones arise by spontaneous mutation;

(ii) the syngen 7 mitochondria aquire the resistance by "recombination" with the micro-injected syngen 1 erythromycin resistant mitochondria, by a mechanism involving the incorporation of an erythromycin-resistant "gene" from syngen 1.

(iii) the syngen 7 sensitive mitochondria are eliminated by erythromycin and the syngen 1 resistant mitochondria replicate to produce mitochondria with their nuclear component from syngen 7 and their mitochondrial component from syngen 1.

The possibility that the syngen 7 cells aquire resistance by spontaneous mutation (i) can be eliminated since it is known that the spontaneous mutation rate is of the order of 1 per 1 000 cells and this is confirmed by the absence of erythromycin-resistant cells amongst the un-injected controls, compared to the higher frequency of aquisition of resistance following micro-injection (1 in 20 cells).

The reduction in frequency of transfer of erythromycin resistance from 70–100% in intrasyngen experiments to about 5% in intersyngen experiments might be explained by a requirement for recombination in intersyngen transfers. Two pieces of evidence make recombination an unlikely possibility. Repeated attempts to obtain recombination between erythromycin and chloramphenicol resistant markers in syngen 4 (Beale *et al.*, 1972; Adoutte and Beisson, 1972) have been made but no recombination has been obtained. This is in marked contrast to results obtained with yeast (Thomas and Wilkie, 1968; Coen *et al.*, 1970; Wilkie, 1970) where recombination between similar markers can be obtained readily. The negative results with *Paramecium* suggest that mitochondrial recombination between these two markers occurs extremely rarely, if at all, in *Paramecium* and suggests that recombination may be a rare event in general.

Preliminary results with resistance transfer between syngens 1 and 5 shows a similar lowered percentage of transfer of resistance when syngen 1 resistant mitochondria are micro-injected into syngen 5 sensitive cells. When resistant mitochondria, prepared from the "hybrid" syngen 1/5 resistant cells are micro-injected into syngen 1 sensitive cells, 70–100% transfer of resistance is observed. This implies that the syngen 1 resistant mitochondrial genome is unchanged after replicating within the syngen 5 cell. If resistant mitochondria are prepared from the syngen 1 cells which have been previously micro-injected with mitochondria from the "hybrid" syngen 1/5 cells and then micro-injected back into syngen 5 sensitive cells, the same lowered percentage transfer of resistance is observed as that observed initially. If recombination between the mitochondrial genomes of syngens 1 and 5 had occurred one would expect an increased percentage resistance transfer back into syngen 5. Further work is in progress to establish these findings more definitely.

Thus, the most likely explanation for the results described here is that the whole syngen 1 mitochondrial genome is transferred into syngen 7 cells. The reduced frequency of transfer is possibly due to the interaction of the syngen 7

nuclear genome and the syngen 1 mitochondrial genome. It is hoped that by extending the search for mitochondrial protein differences between syngens, this approach will provide a valuable contribution to the elucidation of the informational content of mitochondrial DNA.

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Results

Part I: Preliminary studies on the location of erythromycin resistance in Paramecium aurelia by microinjection.

1. To find out if it was possible to transfer this resistance by microinjection a 500 g. supernatant from a homogenised preparation of erythromycin resistant cells was injected into sensitive cells. This supernatant contained most cell components except for macronuclei and large pieces of pellicle. However initial experiments were unsuccessful until the developments in the technique of microinjection described in the last section allowed the routine use of injection needles with larger internal diameters.

Table 1 shows the number of cells becoming resistant following microinjection with a 500 g. supernatant. Of the 42 cells surviving injection, 22 developed resistance to erythromycin.

TABLE 1. Development of resistance in cells injected with a 500 g supernatant

Date	Total number surviving injection	Number Resistant	%
21.10.70.	8	1	12
22.10.70.	8	4	50
29.10.70.	6	3	50
12.11.70.	20	14	70

It should be noted that positive results were only obtained following the use of an injection needle with an internal diameter of not less than 2.8 microns, and that this corresponds well to the size of the mitochondria of paramecium (2-4 microns).

The Development of Resistance

When cells sensitive to erythromycin are placed in bacterised slide medium containing 250 mg/ml erythromycin, they are unable to divide beyond one or two residual fissions. However, these organisms are not killed for some 2 to 4 weeks. During this period they grow slowly and become black and thin. Drug resistant mutants grow at the normal rate in the presence of erythromycin.

Sensitive cells injected with a cytoplasmic extract derived from resistant cells develop resistance in a very characteristic way. Initially they appear to be sensitive to erythromycin. After a variable period of time the cells pass through an intermediate stage, lasting about 12 hours, when they are unusually pear shaped and fast swimming. After this the cell resumes normal growth in the presence of erythromycin and is then classified as having developed resistance.

2. In an attempt to purify the cellular fraction conferring resistance to erythromycin a procedure using a cushion of 50% sucrose in deuterium oxide was developed. This procedure produced a purer preparation mitochondrial/than that obtained with a 5,000 g. pellet and had the advantage of not requiring the prolonged presence of high sucrose concentrations necessitated by the use of equilibrium gradients.

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Figure 2: This shows a section of an osmium fixed pellet of mitochondria prepared on a sucrose step gradient. Most mitochondria are swollen. (X 20,000).

However, it was found that these preparations were unable to transfer erythromycin resistance to sensitive cells (Table 2).

TABLE 2: Development of resistance in cells injected with mitochondria from D_2O/H_2O interface.

Date	Total number surviving injection	Number Resistant
19.11.70.	9	1
20.11.70.	18	0
3.12.70.	9	0

36 cells were injected with this type of preparation and only one developed resistance to erythromycin. Furthermore, these preparations were often lethal on injection unless diluted because of the high concentration of sucrose present.

An electron microscope examination of a preparation of this type showed that although there was little contamination by other cell components, the mitochondria appeared very much more swollen and damaged as compared to mitochondria fixed in vivo (Fig.2) It was felt that these structural changes produced by preparing mitochondria in 50% sucrose might well affect the ability of this preparation to transfer resistance to erythromycin.

3. This failure of mitochondria prepared in 50% sucrose to transfer resistance to erythromycin following microinjection prompted the use of a second method for the preparation of mitochondria. This second method was developed by Preer and Preer (1959) with the modification described earlier.

This method has the advantage of being very rapid, and it does not expose the mitochondria to high concentrations of sucrose. Of the 20 cells injected in the first experiment using this type of preparation, 18 of these cells became resistant to erythromycin. Table 3 shows the experiments in which this type of preparation was used. 82% of the cells injected with this type of mitochondrial preparation developed resistance to erythromycin.

TABLE 3: Development of resistance in cells injected with mitochondrial preparations (5000 g. supernatant).

Date	Total number surviving injection	Number developing resistance
9.12.70.	20	18
5. 6.71.	9	7
5. 6.71.	7	4
19. 6.71.	11	9
20. 6.71.	5	2
24. 6.71.	13	13
5. 7.71.	8	7
6. 7.71.	8	2
9. 7.71.	5	4
6.10.71.	11	11
8.10.71.	8	8
9.10.71.	4	4
21.10.71.	7	7
23.10.71.	9	7
9.11.71.	7	5
7. 7.72.	12	12
5. 7.72.	18	8
30. 6.72.	24	24
25. 6.72.	13	13
2. 2.72.	17	17
15. 2.72.	16	12
Total	232	191

$^{191}/_{232}$ = 82% develop resistance.

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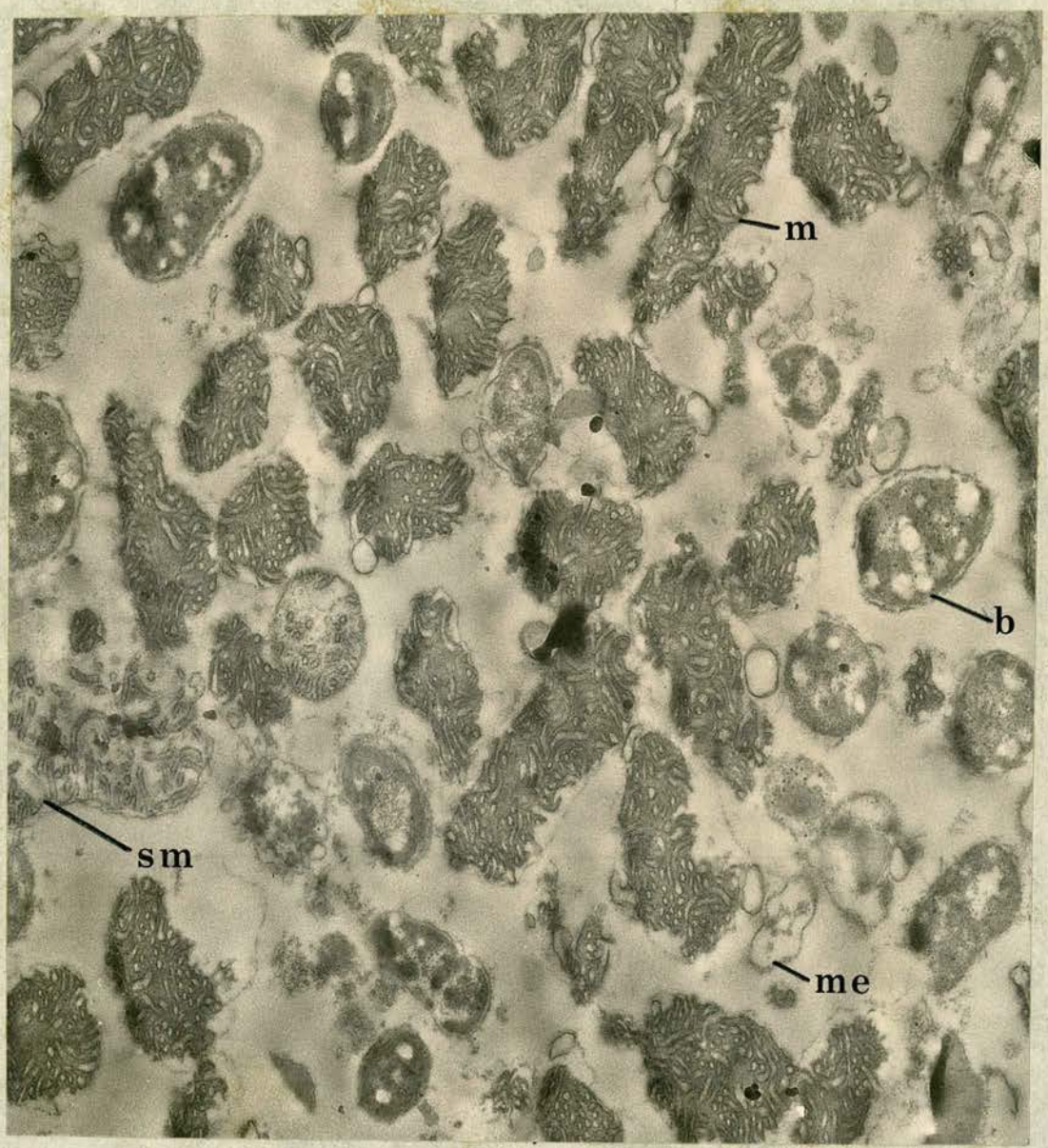

Figure 3: This shows a section of an osmium fixed pellet of a mitochondrial preparation. Note that many mitochondria are highly condensed.

m - mitochondria
b - bacteria
sm - swollen mitochondria
me - membranes

(X 25,000).

4. Control Experiments

(a) Cells were injected with a post-mitochondrial (5000 g) supernatant. Of the 14 cells injected with this cell fraction none developed resistance to erythromycin.

To show that acquisition of erythromycin resistance is not just an artefact of injection, sensitive cells were injected with a preparation of mitochondria from the same clone of sensitive cells. Of the 36 cells injected with this preparation, none became resistant.

Further, of the 1,120 uninjected control cells derived from the same clone as and treated in an identical manner as those injected, not one became resistant.

5. The purity of mitochondrial preparations

An attempt was made to obtain information about the purity of mitochondrial preparations used for microinjection by the examination of pellets of these preparations in the electron microscope (Fig.3). The frequencies of the various components are shown in Table 4.

TABLE 4. The relative numbers of different components in mitochondrial preparations, as judged by electron microscopy.

Component	Numbers counted	%	% of mitochondria
"Normal" condensed mitochondria	899	68	84
Swollen mitochondria	166	12	16
Trichocysts	50	4	
Bacteria	144	11	
Membranes	51	3.5	
Cilia	20	1.5	

It was found that mitochondria made up 80% of the total and could be classified into two classes (Fig.3).

(a) Highly condensed mitochondria with a dense matrix, making up 68% of the total, and,

(b) Swollen mitochondria with a clear matrix, being 12% of the total. Many of these had incomplete inner and outer membranes and some were completely ruptured.

Observation of the mitochondrial preparation by light microscope at periods of up to 7 hours after homogenisation showed an increase in the number of swollen translucent mitochondria. It is probable that these swollen translucent mitochondria are incapable of transmitting resistance to erythromycin.

The major contaminant was bacteria which comprised nearly 11% of the total. Other contaminants present were trichocysts, both exploded and unexploded (4%), Various membranes largely derived from either the pellicle or from the endoplasmic reticulum (4%) and cilia (1.5%).

Part II. The Effect of DNAse, RNAse and Detergent on the ability of mitochondrial preparations to transfer resistance to erythromycin.

This section describes the effects of nucleases and detergents on the ability of mitochondrial preparations to transfer resistance to erythromycin to sensitive cells by microinjection.

(1) Treatment with Detergent

When a mitochondrial preparation was treated with 1.5% Triton X 100 for 30 minutes at 0°C prior to injection, it was found that none of the 15 cells injected with this preparation developed resistance. In contrast, all 13 of the cells injected with a similar but untreated control preparation did become resistant to erythromycin. Light microscope observation of the treated preparation showed that very few mitochondria-like bodies were still visible.

This result shows clearly that even relatively low concentrations of non-ionic detergent completely remove the ability of mitochondrial preparations to transfer resistance.

(2) Treatment with DNAse

The treatment of mitochondrial preparations with 1 mg/ml of DNAse II for 1.5 hours at 0°C prior to microinjection was found to have no

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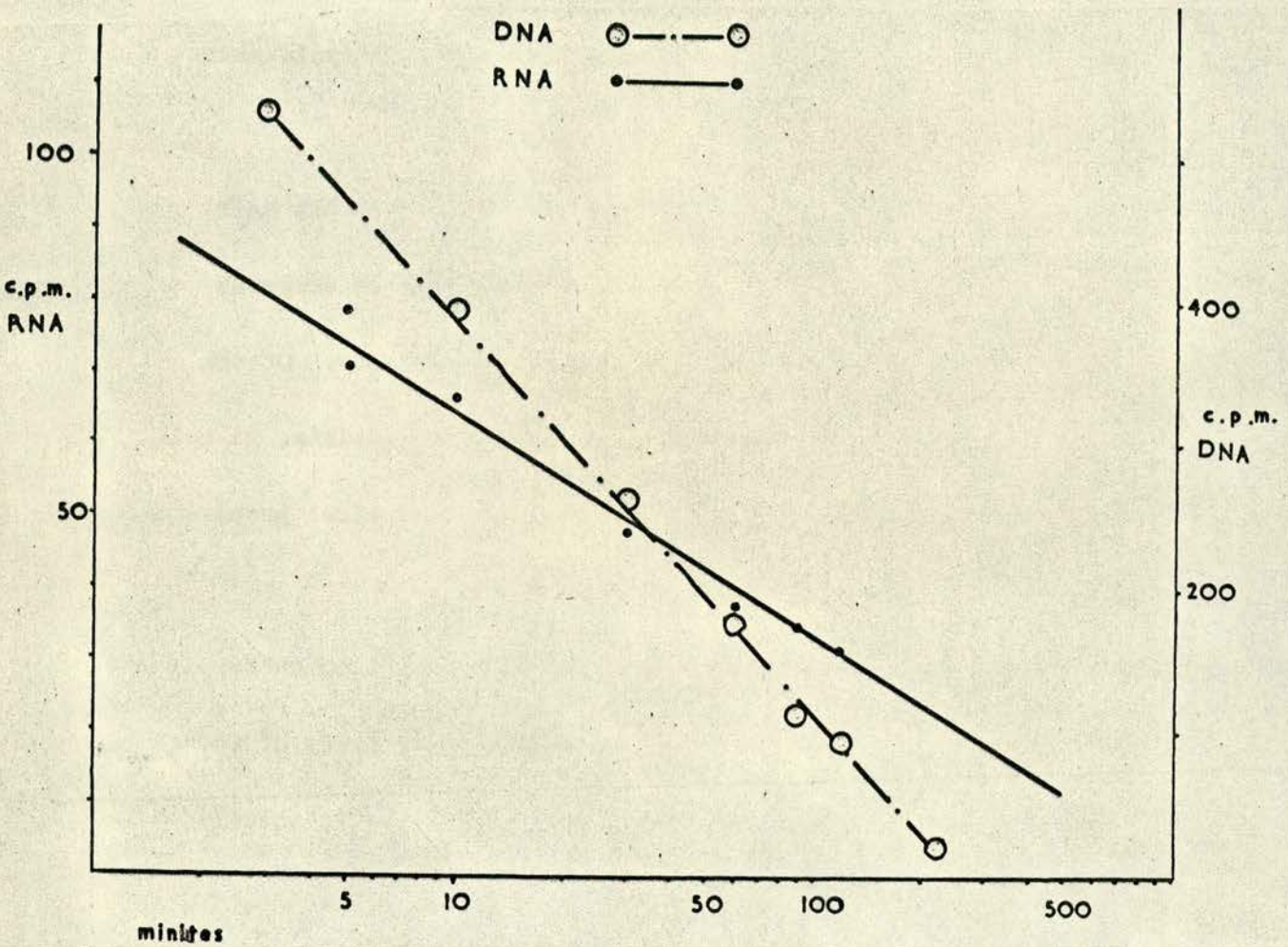

Figure 4: This shows the decrease in T.C.A. precipitable counts with time when ^{14}C DNA is incubated with 1 mg/ml DNAse in rafinose homogenization buffer — . — . — O, and when ^{14}C RNA is incubated with 1 mg/ml RNAse in rafinose homogenization buffer ——— ● ——— . The closest approach to a straight line was achieved by a semi log plot.

measurable effect on transmission of erythromycin resistance to sensitive cells. Of the 28 cells injected with this preparation, 26 developed resistance. In the control group injected with a similar untreated preparation, 28 out of 31 cells developed resistance.

(3) Treatment with RNAse

The treatment of a mitochondrial preparation with 1 mg/ml of RNAse for 1 hour at $0^{\circ}C$ was also found to produce no measurable effect on the transmission of erythromycin resistance. Of the 37 cells injected with treated preparations of mitochondria, 31 cells became resistant, of 33 cells injected with the control preparation 29 developed resistance.

As the composition of the mitochondrial isolation buffer is not similar to that of solutions used to assay the activity of nucleases, experiments were carried out to estimate the catabolic activity of the two nucleuases in the isolation buffer. Radioactive polyribo- or deoxyribonucleic acids, and the relevant nuclease were incubated in the isolation buffer and the rate of degradation estimated by counting trichloroacetic acid precipitable radioactivity at various times. These results are plotted in Fig.4.

These graphs show that the amount of TCA precipitable radioactive DNA is reduced by one half in 45 minutes and the amount of radioactive RNA is reduced by one half in 30 minutes.

These results show that the factor confirming resistance to erythromycin is not affected by DNAse or RNAse but is completely destroyed by low concentrations of non-ionic detergent.

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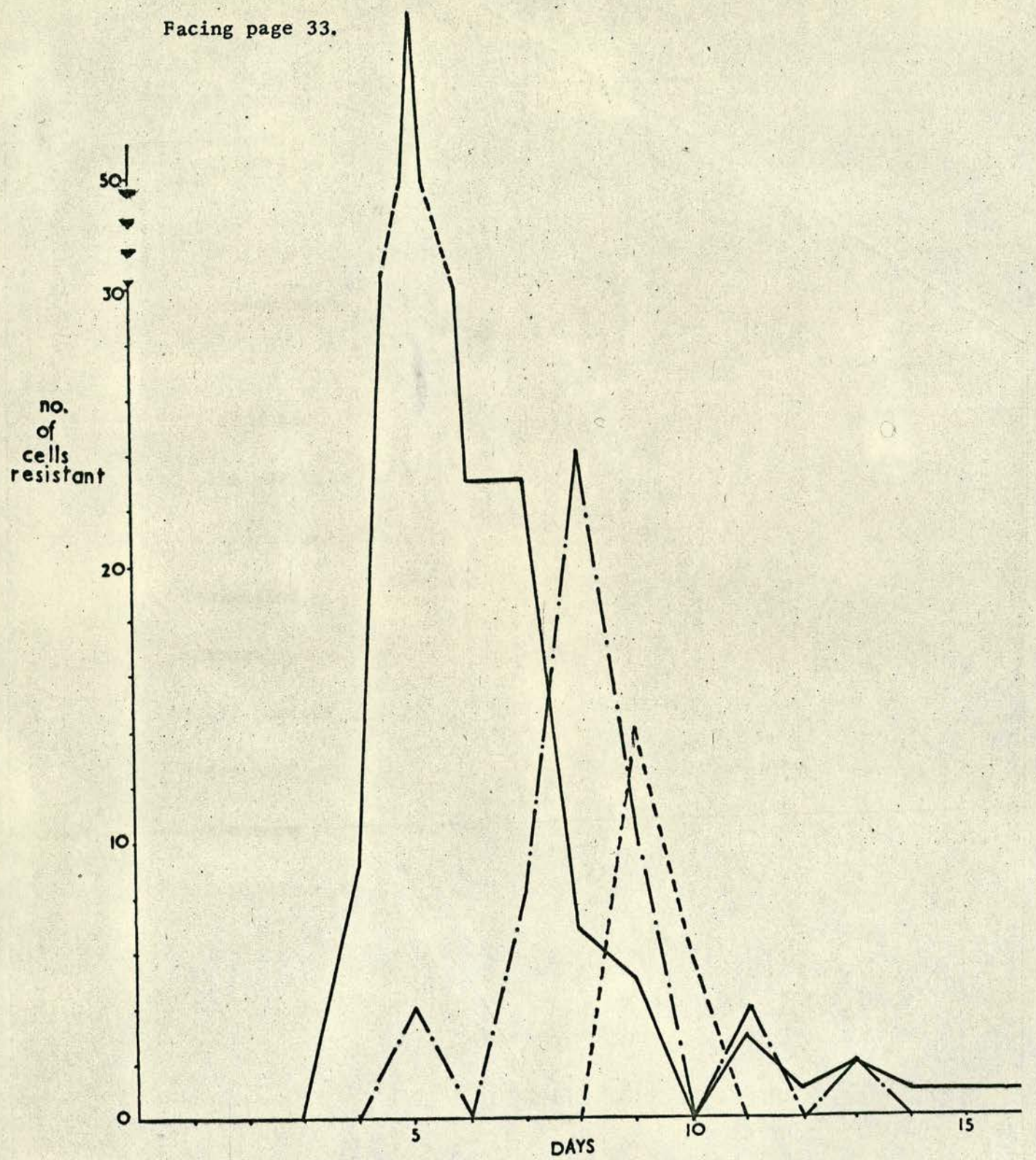

Figure 5: This shows the time taken for cells injected with different numbers of mitochondria to develop resistance.

Cells injected with:
——— more than 50 mitochondria.
— . — a mean of 5 mitochondria per injection.
- - - - - a mean of 1 mitochondria per injection.

Part III. The effects of injecting small numbers of mitochondria into single cells.

(1) The effect of injecting different numbers of mitochondria on the time taken to develop resistance.

It should be noted that the results to be discussed here are preliminary and can at best only give an indication of the effects of injecting small numbers of mitochondria into cells.

If the development of resistance to erythromycin following microinjection of a sensitive cell is defined as having occurred when the number of cells in any depression exceeds twelve, it is possible to record with an accuracy of ± 24 hours the time taken to develop resistance. In these experiments the initial concentration of mitochondria was estimated by means of a special counting chamber and the original preparation diluted to give a mean of one or five mitochondria per injection. Figure 5 and Table 5 show the time in days for cells to develop resistance following injection with either a mean of 1 or 5 mitochondria per injection. The control cells shown are those cells injected with between 50 and 100 mitochondria per injection.

TABLE 5: The time taken for cells injected with different numbers of mitochondria to develop resistance.

Day	50/injection	5/injection	1/injection
1			
2			
3			
4	9		
5	56	2	
6	23		
7	23	4	
8	7	12	
9	5	5	7
10	0		3
11	3	2	
12	1		
13	2	1	
14	1		
15	1		
16	1		
17	1		
18			
19			
20			

The median time taken for cells injected with a mean of one mitochondria to develop resistance is 9 days, whereas cells injected with an average of 5 mitochondria develop resistance in a median time of 8 days. The control cells injected with more than 50 mitochondria have a median time of 5 days required to develop resistance.

Although preliminary, these results indicate that the time taken by a cell to develop resistance is roughly proportional to the absolute numbers of mitochondria from a resistant strain injected into that cell.

(2) The effect of the numbers of mitochondria injected on the frequency of development of resistance.

(i) Of the 35 surviving cells injected with a mitochondrial preparation diluted to give an average of 1 mitochondrion per injection, a total of 10 became resistant

(ii) Of the 38 surviving cells injected with a preparation diluted to give 5 mitochondria per injection a total of 26 developed resistance.

(iii) Results already presented (Table 3) show that on average 82% of the cells injected with more than 50 mitochondria develop resistance.

The significance of these results should be examined by comparing them with the expectations for the number of "successes" derived from a poisson distribution and this will be discussed fully in the next (Discussion) section. However, they do suggest that very few mitochondria are required to change the phenotype of a sensitive cell to erythromycin resistance.

Discussion

The aim of the work presented in this chapter was to locate the site of cytoplasmically inherited erythromycin resistance in *Paramecium aurelia*.

The evidence presented in Section I of the Results shows that the cell fraction most efficient at transferring resistance to erythromycin to sensitive cells was the mitochondrial fraction prepared by differential centrifugation. It was shown by electron microscope observations of sectioned mitochondrial pellets that these preparations contained about 80% mitochondria. The major contaminant was bacterial, but the bacteria in these preparations had never been exposed to erythromycin and were found to be sensitive to erythromycin (West, unpublished).

Attempts to induce development of erythromycin resistance by injection with purer fractions of mitochondria prepared with sucrose step gradients were unsuccessful. This failure was probably due to damage to mitochondria caused by the high concentrations of sucrose used. Rat liver mitochondria are partially disrupted by the hydrostatic pressure produced during rapid centrifugation (Wattiaux et al., 1971), and a similar effect may also have damaged *Paramecium* mitochondria prepared on sucrose step gradients. Examination of sections of mitochondrial pellets produced by sucrose step gradients showed that the mitochondria appeared very much more disrupted than those prepared by differential centrifugation.

In Neurospora, Diakumacos et al. (1965) were able to obtain transmission of a cytoplasmic character causing slow growth, abn 1, by microinjecting abn 1 mitochondria prepared on a continuous sucrose gradient into normal hyphae. It seems likely that there are differences in the sensitivity of mitochondria from Paramecium and Neurospora to high osmotic and hydrostatic pressures and this may be due in part to the two-fold differences in mitochondrial size between these species.

Examination by electron microscopy of fixed pellets of mitochondria prepared by differential centrifugation showed that the great majority of these mitochondria were very much more condensed than mitochondria examined in sections of whole cells. Hakenbrook (1968, 1972), Penniston et al. (1967) and Green and Harris (1969) have shown that inhibition of certain parts of oxidative phosphoration in rat liver mitochondria produces a condensed and densely staining matrix. The extremely condensed appearance of mitochondria in the preparations used for microinjection may be due to inhibition of part of oxidative phosphorolation by the low oxygen tension in these concentrated suspensions of mitochondria. However, this abnormal physiological condition does not seem to affect the ability of these organelles to transfer resistance to erythromycin into sensitive cells.

Part II of the Results presents evidence that neither DNAse nor RNAse appears to have any measurable effect on the ability of mitochondrial preparations to transfer resistance to erythromycin, while low concentrations of Triton X 100 completely removed this ability.

If the determinant for erythromycin resistance is located on the mitochondrial DNA, the nucleases would not be expected to have an effect on the transmission of erythromycin resistance, because this DNA is protected by the mitochondrial membranes. Flavell and Jones (1971) have shown in Paramecium that there is little reduction in the amount of mitochondrial DNA obtained when the mitochondria are pretreated with DNAse.

The primary effect of the detergent appears to be the destruction of the gross structure of the mitochondria. Whether the whole structure of the mitochondria is required for transmission of erythromycin resistance is not known, as it is possible that the mitochondrial DNA is rapidly degraded on the breakdown of the mitochondrial membranes. The inability of nucleases to affect the transmission of erythromycin resistance does rule out the possibility that this transmission is caused by extra-mitochondrial DNAs or RNAs bound to the surface of the organelle. It is of interest to note that the use of a needle with an internal diameter too small to allow passage of whole mitochondria also prevents transmission of resistance to sensitive cells even though the injection of normal quantities of fluid occurs.

Part III of the Results present preliminary evidence suggesting that only one mitochondrion may be required to transform a previously sensitive cell to erythromycin resistance. However, in the analysis of these results the following three factors should be taken into account.

1) Only about 84% of the mitochondria present in mitochondrial preparations are structurally intact as judged by examination of their membranes in the electron microscope.

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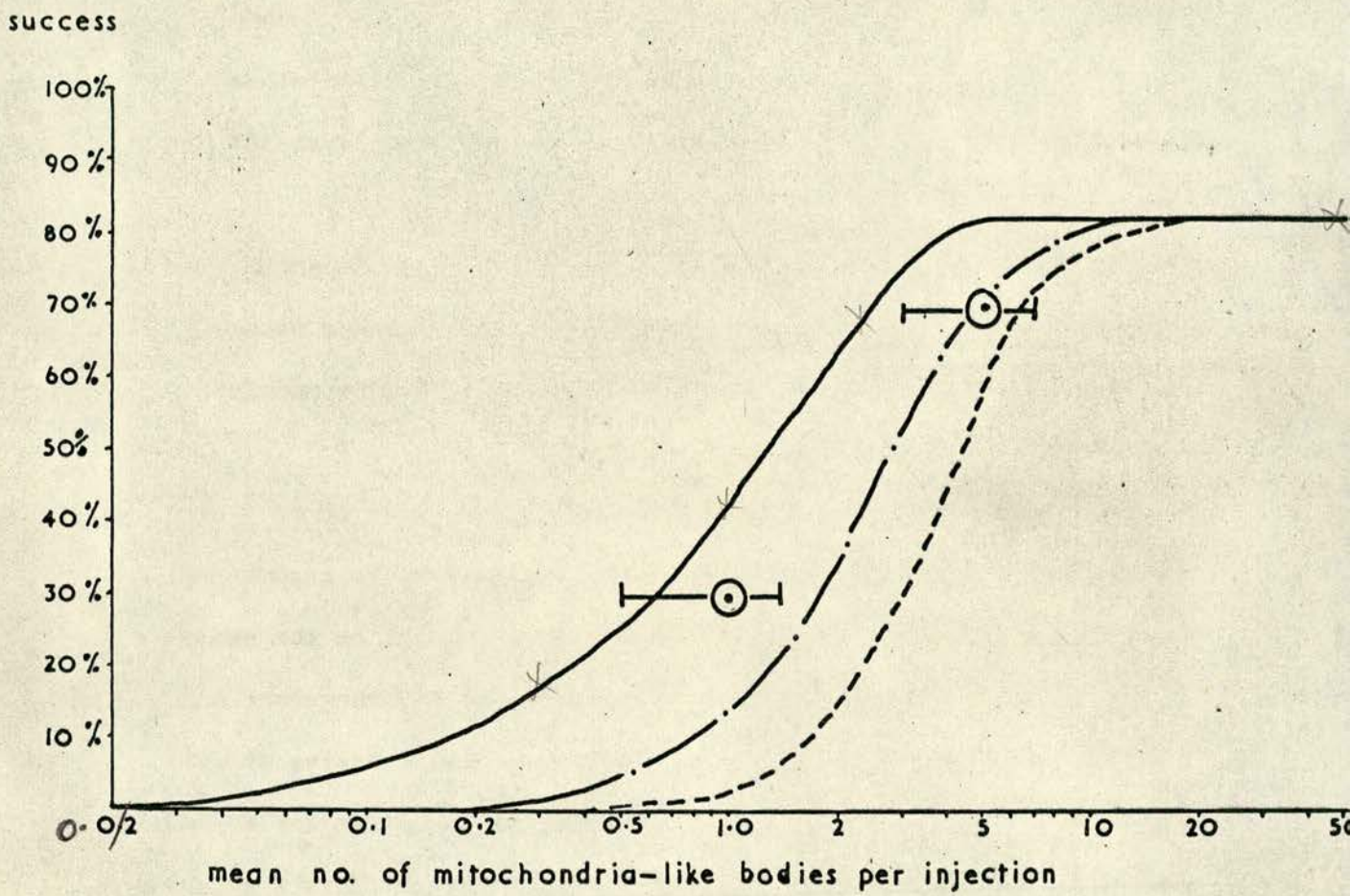

Figure 6: These graphs show the percentages of the total number of cells injected which would be expected to develop resistance if one (———), two (— . — . —), and three (- - - - -) mitochondria were sufficient to transfer erythromycin resistance for a given mean number of mitochondria per injection. The two experimental results are also shown with ± 1.96 standard deviation errors.

2) It is difficult to distinguish between Klebsiella aerogenes, the food organism, and Paramecium mitochondria with a light microscope.

These two factors make estimates of the number of mitochondria capable of transmitting resistance to erythromycin at least 15% too high.

3) Even in experiments where sensitive cells are injected with more than 50 mitochondria per injection only 82% of these become resistant. Therefore, any estimate of success should be modified such that the maximum possible success is 82%.

As the volume of the mitochondrion is of the order of 10^{-4} of the volume injected into each cell, it is reasonable to assume that the presence of one mitochondria has negligible effect on the presence or absence of any other. A poisson distribution may therefore be used to predict the proportion of injections which transfer at least one, at least two, at least three, ... etc. mitochondria for a given mean number of mitochondria per injection. Figure 6 shows a graph of three curves each one representing the percentage of cells receiving at least 1, at least 2, and at least 3 mitochondria per injection for a range of mean numbers of mitochondria per injection. The poisson expectations on the graph have been modified in the following way:

Factor 1 (0.84) and Factor 2 (0.90) affect the mean number of "viable" mitochondria per injection and so this mean number is divided by 0.84 x 0.90. Factor 3 reduces the expectation of success and so is applied to the probability that a particular cell will receive at least 1, 2 or 3 mitochondria.

It should be noted that these three factors represent only those effects which are roughly quantifiable, and so the modified expectations are probably still an overestimate of the number of cells expected to develop resistance.

When the two preliminary results obtained so far are plotted on this expectation graph they both fell between at least 1 and at least 3 mitochondria required to transfer resistance to erythromycin. This evidence shows that only a very few mitochondria are required to transfer erythromycin resistance to a sensitive cell. If this finding was confirmed it would provide conclusive evidence that most if not all the mitochondria in one cell were genetically equivalent with respect to erythromycin resistance.

In summary, by microinjection of various cell fractions into sensitive cells it has been shown that erythromycin resistance is located in the mitochondria, probably on the mitochondrial DNA. The possibility that some other cell fraction could be responsible for transmitting erythromycin resistance has been ruled out for two reasons. Firstly, the post-mitochondrial supernatant was shown to be unable to transmit erythromycin resistance to sensitive cells. Secondly, while low concentrations of non-ionic detergent completely removed the ability of mitochondrial preparations to transfer erythromycin resistance, RNAse and DNAse were found to have no effect. In the course of this work the technique of microinjection of Paramecium has been developed to a state where large numbers of cells can be injected with relative ease.

CHAPTER II

A Study by Electron Microscopy of Transformation from Sensitivity to Erythromycin Resistance Following Microinjection.

Introduction

Paramecia collected from nature are sensitive to erythromycin; when they are placed in bacterized grass medium containing o.25 mg/ml erythromycin and incubated at 25°C, cell division is blocked after one or two residual fissions. However, the cells are not killed for 2-4 weeks, but swim slowly and become black and thin. Drug resistant mutants have been obtained (Beale, 1969; Adoutte and Beisson, 1971, 1972; Beale et al. 1972), and these grow at the normal rate in medium containing the drug.

When sensitive paramecia are injected with a preparation of mitochondria from resistant cells and these injected cells are placed in erythromycin containing medium, resistance to erythromycin usually develops within a period of between 4 and 15 days (Beale et al., 1972; Chapter I). Before becoming completely resistant, the cells pass through an intermediate stage when they are pear-shaped and fast swimming.

This chapter describes an investigation of the process by which cells, injected with mitochondria from a resistant clone, develop resistance to erythromycin when placed in selective medium. In contrast to earlier studies on mitochondrial genetics which were based

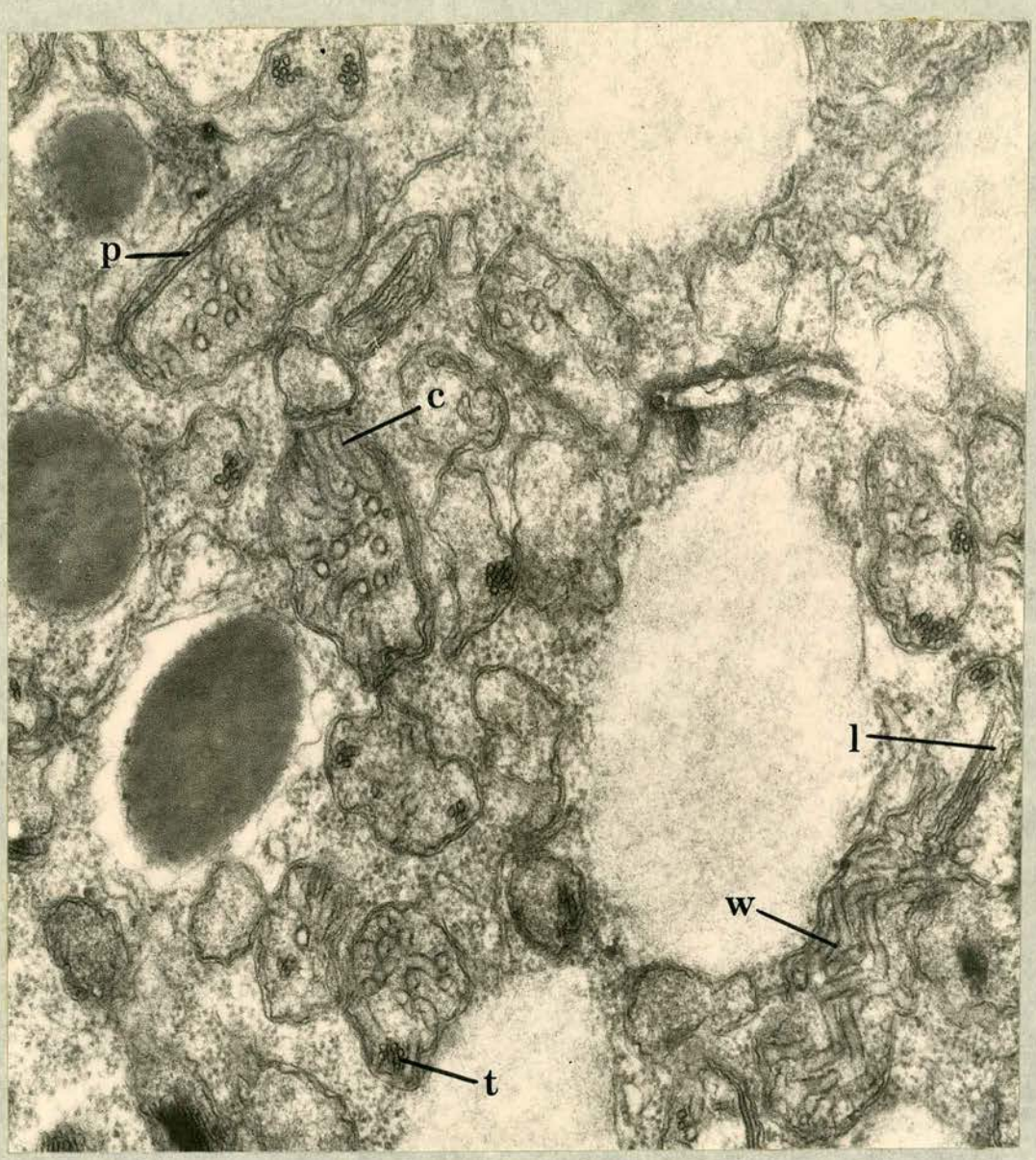

Figure 7: Mitochondria in a sensitive cell exposed to erythromycin for 4 days. This shows:

a) loss of cristae (compare with Fig.8)

b) wavy conformation of cristae (w).

c) flat plate like lamellae (p) parallel to the outer membrane.

d) laminar cristae, distinguished from (c) by variation in the distance between the two component membranes. (1)

e) bundles of round tubules (t) about 20 nm in diameter.

(X 50,000)

Figure 8: Mitochondria of aw wild type sensitive cell in the absence of antibiotic, showing normal appearance of Paramecium mitochondria. These mitochondria are rounded or slightly elongated, with numerous curved tubular cristae and little matrix. (X 50,000).

Figure 9: Mitochondria in a sensitive cell exposed to erythromycin for 6 days. This shows the greatly elongated mitochondria containing few cristae but several bundles of tubules and rigid plates. (X 50,000).

on observations of whole cells, the work described here considers the effect of erythromycin on individual mitochondria within a cell, as seen by electron microscopy.

Results

To eliminate variation of culture conditions, all the observations described in this chapter were made on cells which had been grown in depression slides and embedded and sectioned individually.

1. The Effect of Erythromycin on Sensitive Cells.

After exposure of sensitive paramecium to 250 µg/ml erythromycin, the following modifications of mitochondrial structure were noted, (Figs. 7 and 9).

a) Cristae were lost progressively with time in almost all of the mitochondria examined when compared to untreated cells, (Fig.8). The remaining cristae often form a repetitive wavy structure.

b) The shape and size of the mitochondria become irregular. In particular there is a marked reduction in the diameter and an increase in length of many of the mitochondria examined.

c) Flat lamellae in the shape of plates of rigid appearance, 25 nm thick, similar to those described by Newcomb et al., (1968) in bean root mitochondria. These rigid plates often exhibit a periodic structure.

d) Bundles of round rod like "tubules" about 20 nm. in diameter, quite distinct from cristae (diameter 40 nm.). The number of tubules in a bundle may vary but the diameter of the tubule is constant.

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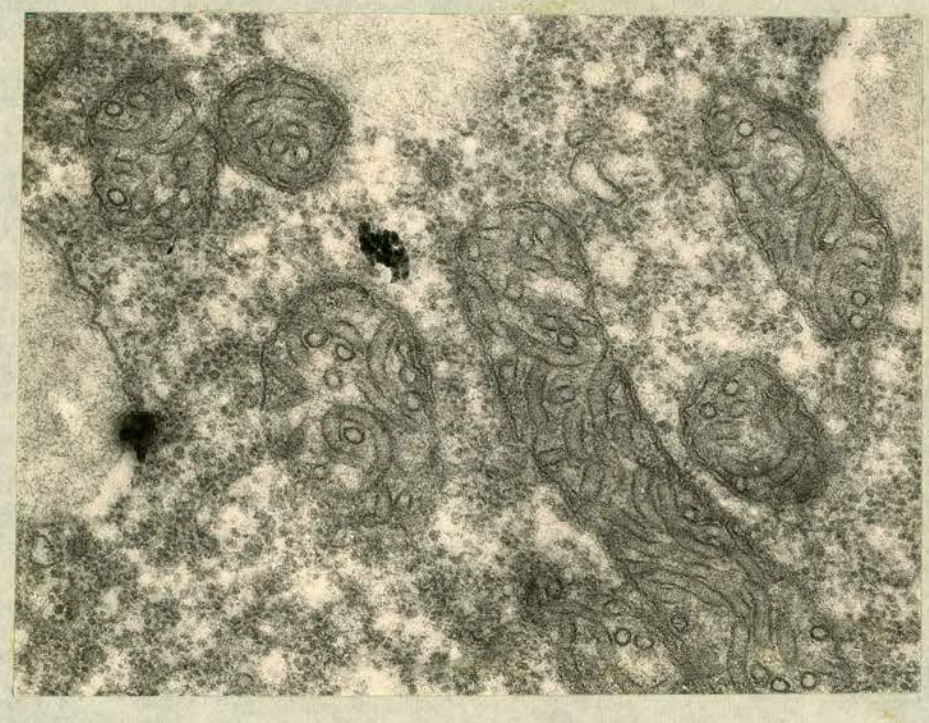

Figure 10: Mitochondria in a resistant cell from a clone grown in selective medium for 9 days. These mitochondria are indistinguishable from those observed in untreated sensitive cells. (X 50,000).

e) Laminar cristae are often found next to the mitochondrial inner membrane. These are distinguished from the flat lamellae by their apparent lower rigidity and the variability in the distance between the two component membranes.

It should be noted that in almost all of these abnormal mitochondria, even those completely lacking cristae, both outer and inner mitochondrial membranes appear to persist. In addition no other cellular structure appears to be modified; cell cortex trychocysts and nuclei appear normal. The primary effects of erythromycin seem to be largely restricted to the mitochondrial cristae.

These conclusions are based on the examination of 76 sections from 28 individual cells.

2. The Effect of Erythromycin on Resistant Paramecia.

Erythromycin at a concentration of 250 μg/ml had no observable effect on the resistant mutant after 6 days of growth in the drug (Fig.10). The mitochondria in sections of these cells were indistinguishable from mitochondria in untreated paramecia (Fig.8). This conclusion is based on examination of 24 sections of 18 cells.

3. The Effect of Erythromycin on "transforming" cells produced by microinjection.

Cells which had become pearshaped and fast swimming after microinjection with mitochondria from the resistant strain were fixed approximately 24 hours before their first division. These cells were found to contain mitochondria of three types.

Figure 11: This shows the presence of two mitochondrial phenotypes in one cell "Resistant" (r) and sensitive (s) mitochondrial forms in a previously sensitive cell which had become "pear shaped" and fast-swimming after 13 days in selective medium. (X 50,000).

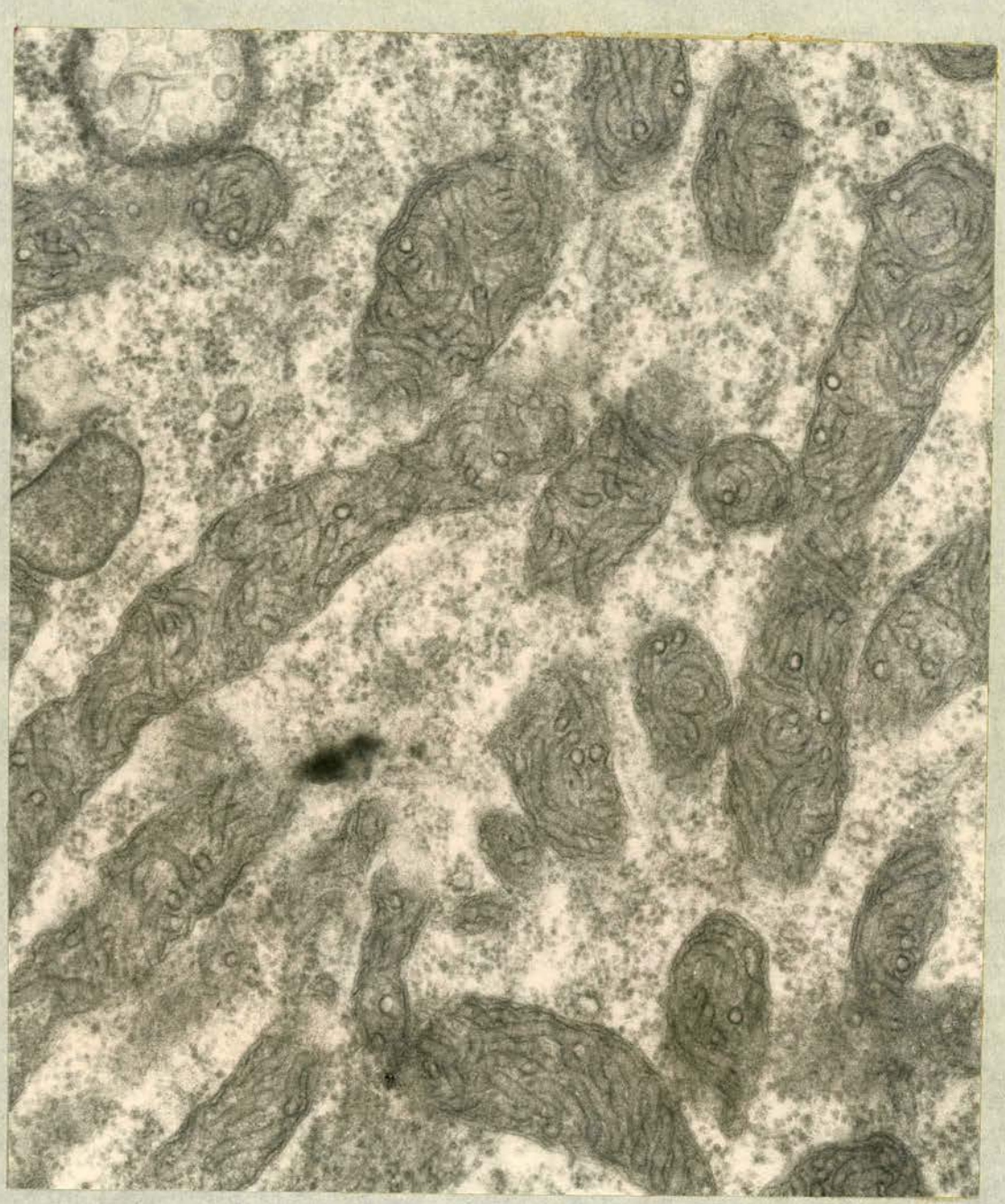

Figure 12: A section through a group of long "resistant" mitochondrial forms from a cell transforming to resistance to erythromycin after 13 days. These mitochondria may well be dividing rapidly. (X 50,000).

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Figure 13: An "intermediate" form in a "transforming" cell placed in erythromycin for 13 days. This contains many normal cristae (c) and also a flat lamella plate (P) similar to those found in sensitive exposed to erythromycin. (Figs. 7 and 8) (X 75,000).

a) Those (presumably resistant) which were apparently unaffected by erythromycin. These could be subdivided into two classes as follows:

(i) Mitochondria having normal dimensions similar to those found in resistant cells (Figs. 10 and 11).

(ii) Mitochondria which appeared considerably longer than those found in normal cells. These often tended to occur in groups (Fig.12).

b) Those mitochondria which were similar in appearance to mitochondria in sensitive cells exposed to 250 μg/ml erythromycin for more than four days.

c) "Intermediate" forms, containing many normally developed cristae but also a flat lamella plate similar to those found in mitochondria of sensitive cells treated with erythromycin (Fig.13). These were observed at a very low frequency (1%).

No other class of mitochondria was observed in these transforming cells. These conclusions were based on the observation of 63 sections of fifteen transforming cells.

To obtain an estimate of proportions of the three mitochondrial types in transforming cells, entire sections from five cells, taken from three separate transformation experiments were photographed and the different classes of mitochondria counted under a binocular microscope. The frequency of these different classes is shown in Table 6.

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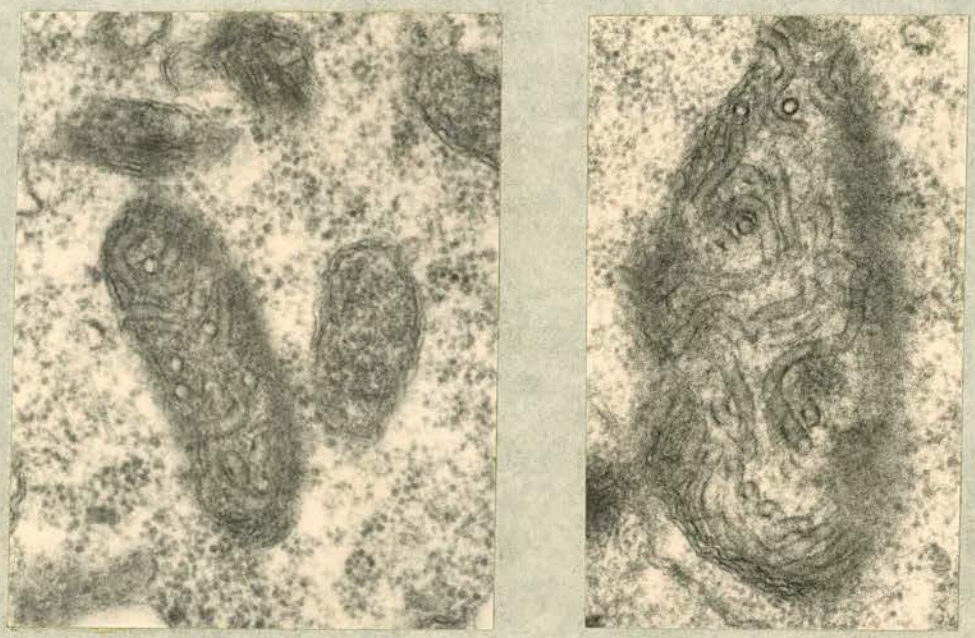

Figure 14: This shows two mitochondria forms from sections of cells which had been injected with mitochondria and incubated for 6 days in erythromycin containing medium. It is possible that these forms are "resistant" to erythromycin in view of the large number of cristae they contain. (X 50,000).

TABLE 6: Relative Proportion of Different Types of Mitochondria in Transforming Cells.

Cell	Total No.of mitochondrial sections counted	Mitochondrial Types % resistant	% sensitive	% intermediate
A	394	77	22	1
B	382	78	21	1
C	638	73	26	1
D	303	81	18	1
E	184	86	13.5	0.5

In view of the similarity in the proportions of the 3 mitochondrial types in the five cells studied, it is of interest to point out that these cells were selected on the basis of their "pear shape" and speed of movement. This suggests that at least 70-80% of the mitochondria must appear "resistant" to erythromycin before any change in cell phenotype is observed.

A preliminary attempt was made to follow the change from erythromycin sensitivity to erythromycin resistance after microinjection by examining cells fixed 4 and 6 days after injection. Observations made on 23 cells proved inconclusive. Almost all the mitochondria examined appeared to be similar to those found in uninjected sensitive cells exposed to erythromycin. Fig.14 shows two mitochondria which do appear unaffected by exposure to erythromycin and it is possible that these are derived from the 'resistant' mitochondria injected into the cell. However, the possibility also exists that these mitochondria may be part of the original sensitive population of the cell.

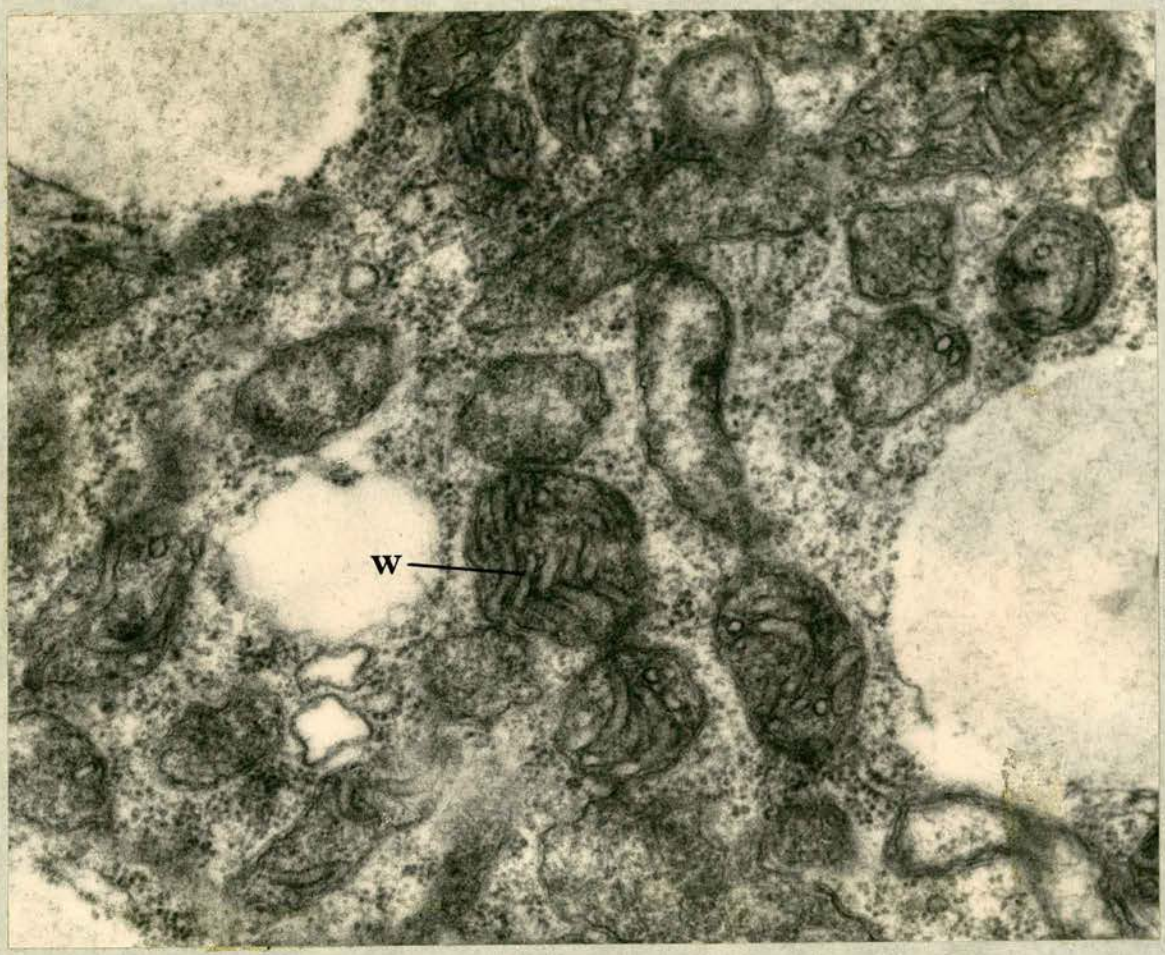

Figure 15: A section of a sensitive cell treated with erythromycin for 12 days and then allowed to recover in non-selective medium. This shows the partial redevelopment of cristae in some of these mitochondria. Some cristae appear to be arranged in a wavy configuration (w) similar to that found in mitochondria exposed to erythromycin for 4 days (Fig.7). (X 50,000).

It appears to be difficult to locate or distinguish the "resistant" mitochondria injected until the change in mitochondrial population is well advanced.

4. The regeneration of sensitive cells exposed to erythromycin for 12 days.

To compare transforming cells resuming growth in selective medium after microinjection, with sensitive cells resuming growth in non-selective medium after treatment with erythromycin, 72 sensitive cells were placed in depressions containing selective medium for 12 days. 43 of these died within 24 hours of transfer to non-selective medium. The 27 surviving cells were fixed for electron microscopy after four days in non-selective medium. By this stage many of the cells had resumed active swimming and resembled the "pear shaped" cells about to develop resistance after microinjection.

Examination of sections of these regenerating cells showed that the mitochondria contained a very variable number of cristae (Fig.15). There was also a considerable reduction in the number of flat lamellae and bundles of round tubules compared with the mitochondria found in sensitive cells incubated in erythromycin for 6 days (Fig.10). However, other mitochondria examined had not regained any visible internal structure. This may well indicate that these organelles had been permanently damaged by the prolonged presence of erythromycin.

It is interesting to note that cristae were often found in a wavy configuration (Fig.15), similar to that observed during initial cristae loss produced by erythromycin.

Thus, although cells developing erythromycin resistance and cells recovering in non-selective media from prolonged exposure to erythromycin are superficially similar in shape and behaviour, the completely different appearance of the mitochondria contained within these two classes of cells strongly suggests that the "transforming" cells do contain two distinct genetic types of mitochondria.

Discussion

1. The Effects of Erythromycin on Sensitive Cells

The results presented in this chapter show that erythromycin has striking effects on mitochondria structure in sensitive cells of Paramecium, while the mitochondria of resistant mutants appear unaffected. The results are in good agreement with those of Adoutte et al. (1972) who studied the effects of erythromycin on resistant and sensitive stocks of Paramecium aurelia Syngen 4.

It is now well established that anti-bacterial antibiotics such as erythromycin and chloramphenicol specifically block mitochondrial protein synthesis without affecting cytoplasmic protein synthesis (Mager, 1960; Kroon, 1965; Wintersberger, 1965; Clark-Walker and Linnane, 1966; Firkin and Linnane, 1968; Lamb et al., 1968; Perlman and Penman, 1970). The inhibition of mitochondrial protein synthesis produces several characteristic biochemical and cytochemical alterations in the mitochondria of cells exposed to these inhibitors. In yeast (Saccaromyces) Clark-Walker and Linnane (1966) have shown that chloramphenicol causes loss of some cytochromes and the disorganisation of the inner mitochondrial membranes. Mahler et al. (1971) using shorter doses of chloramphenicol during respiration derepression concluded that only the synthesis of cytochrome oxidase was affected initially. Smith-Johannsen and Gibbs

(1970) observed loss of cristae from the mitochondria of Ochromonas danica after treatment with chloramphenicol. The same phenomenon has also been reported in HeLa cells by Lenk and Penman (1971). In Tetrahymena, chloramphenicol causes no cytochrome loss initially, but respiration is inhibited and the mitochondria divide to become smaller in size (Turner and Lloyd, 1971).

The primary effect of erythromycin in Paramecium also appears to be the inhibition of protein synthesis by mitochondrial ribosomes (Tait, 1972; personal communication).

The results presented in this chapter show that one of the secondary effects of erythromycin on sensitive Paramecium is to cause large morphological abnormalities in the mitochondria. This may be explained in the following way. Radioactive amino acid incorporation studies (Roodyn et al., 1962) have shown that under appropriate conditions, isolated mitochondria are able to synthesize protein. Fractionation of mitochondria after incubation with radioactive amino acids has shown that incorporation occurs into the membrane protein but not into soluble proteins (Roodyn, 1962; Wheeldon and Lehninger, 1966; Haldar et al., 1966). Some authors have reported that most of the label is in one or two components (Birkmayer, 1971; Kadenback, 1971) but others have found labelled products in up to ten bands of Neurospora (Sebald et al., 1971; Swank et al., 1971; Wolf et al., 1971) Saccaromyces (Weislogel and Butow, 1970; Yang and Cripole, 1969; Thomas and Williamson, 1971) and Tetrahymena (Chi and Suyama, 1971).

The variation in the number of bands is probably due to technical difficulties of the extraction and analysis of these hydrophobic membrane proteins. Identification of the functions of these membrane proteins has proved difficult, but recently a component of cytochrome oxidase (Weiss et al., 1971) and of rutamycin-sensitive ATPase (Tzagoloff and Meagher, 1971) have been shown to be synthesized on mitochondrial ribosomes.

Mahler et al. (1971) have proposed that the effect of antibacterial antibiotics on the structure and composition of mitochondria is probably due to inhibition of mitochondrial protein synthesis which would prevent the production of one or more proteins necessary for the correct assembly of functioning cristae within the mitochondria. According to this hypothesis, no effect would be observed initially in the presence of the drug. However in the absence of synthesis, dilution of these proteins required for the assembly of inner membranes by continued replication and normal degradation would occur. Lower concentrations of these "organizational" proteins would make the formation of normal mitochondrial membranes less and less likely.

The observations reported in this chapter of the effects of erythromycin on the mitochondrial structure of Paramecium are in complete accord with this hypothesis, although the exact nature of the "organizational" protein remains to be defined. The considerable modifications of mitochondrial structure observed under different energy states (Hakenbrook, 1968, 1972; Green and Harris, 1969) and under different nutritional regimes (Daniels and Breyer, 1968) suggest

that mitochondrial membranes may well be paracrystaline in nature, in that different types of stable structure may form depending on the composition of the membrane. An alteration in the composition of mitochondrial membranes caused by inhibition of mitochondrial protein synthesis would be expected to produce new types of paracrystaline structure like the lamellae plates and the bundles of tubules described earlier in this chapter.

It should be pointed out that the abnormal physiological state of sensitive cells exposed to erythromycin or perhaps some indirect effect of the antibiotic on the respiratory chain could also produce changes in the morphology of mitochondria observed. However, unless the mutation to erythromycin resistance affects a wide range of mitochondrial functions, the finding that mitochondria from resistant cells are largely unaffected by erythromycin makes this indirect hypothesis less plausible.

2. The Nature of the change of cell phenotype to erythromycin resistance following microinjection.

The results presented earlier show that two mitochondrial phenotypes exist in one cell during transformation from erythromycin sensitivity to resistance. This finding strongly implies that within the same cell mitochondria are autonomous with respect to each other for erythromycin resistance. Adoutte and Beisson (1972) were able to show the differential elimination of one cytoplasmic marker with respect to the other from cells known to contain two types of mitochondria. They established an order of elimination which was found to

correspond to the temperature sensitivity of the antibiotic resistance markers studied. This evidence also supports the idea of mitochondrial autonomy with respect to antibiotic resistance.

The exact cause of the selective differential between erythromycin-resistant and sensitive mitochondria in one cell is unknown. It was argued earlier (p.49) that the action of erythromycin on "sensitive" mitochondria was to alter the composition and properties of the inner mitochondrial membrane. The DNA of mitochondria is most probably closely associated with the inner membrane. If this modification of the inner membrane also affected the rate of replication of the mitochondrial DNA in "sensitive" mitochondria while the rate of replication of mitochondrial DNA in "resistant" mitochondria remained unaffected, a cell containing both types of mitochondria would become populated only with "resistant" organelles when placed in erythromycin.

More specifically, Williamson et al. (1972) have shown that Saccharomyces cerevisiae exposed to high concentrations of erythromycin and chloramphenicol develop a high frequency of cytoplasmic petites. Mitochondrial DNA was synthesized normally during the silent pre-mutational period, but was rapidly lost by a process partly dependant on degradation, at the time of the mutational event. They have interpreted these findings as evidence that the normal maintenance of mitochondrial DNA in this organism requires a specific protein or proteins whose assembly is inhibited by high concentration of erythromycin. The absence of this protein would produce a selective differential between "erythromycin resistant" and "erythromycin sensitive" mitochondria in paramecium.

It should be pointed out that the erythromycin concentrations required to induce petites are much higher than those normally used to select for erythromycin resistance in both yeast and paramecium. It is more probable that the selective differential between the two types of mitochondria is of a partial nature and does not involve the complete loss of DNA from "sensitive" mitochondria. This idea is supported by the observation that some mitochondria exposed to erythromycin for 12 days can regenerate cristae when placed in drug free medium.

It is also possible that previously 'sensitive' mitochondria might become 'resistant' to erythromycin by acquiring part or all of a genome conferring resistance from one of the "resistant" mitochondria injected into the cell. The occurrence of low numbers of "intermediate" mitochondria observed in transforming cells does not conflict with this hypothesis.

To examine the possibility that the modifications of mitochondria structure observed in transforming cells were due to the abnormal physiological conditions in the cell, sensitive Paramecia were exposed to erythromycin and then allowed to recover in non-selective media. Cells recovering from this long dose of erythromycin were similar in external appearance to cells transforming to resistance following microinjection. However, in marked contrast to the two major classes of mitochondria found in transforming cells, the regenerating cells were found to contain a wide range of mitochondria in different stages of regeneration (or degeneration).

It is therefore most likely that the presence of three different classes of mitochondria observed in transforming cells is correlated with the presence of at least two genetically different types of organelle.

In summary, results reported here show firstly that erythromycin has striking effects on mitochondrial structure in sensitive cells of Paramecium, and secondly that in cells transforming to erythromycin resistance following microinjection, two mitochondrial phenotypes can be observed within the same cell.

These findings have been interpreted as evidence that transformation of cell phenotype to erythromycin resistance occurs through the process of differential replication of the injected "resistant" mitochondria, With respect to the original population of "sensitive" organelles.

CHAPTER III

The transfer of mitochondria between syngens

Introduction

The technique of microinjection of sensitive cells with preparations of mitochondria from resistant cells, described in Chapter I allows the transfer of mitochondria between genetically isolated syngens and species of Paramecium. This approach makes possible investigation of two important aspects of mitochondrial genetics. Firstly, information concerning the ability of erythromycin-resistant mitochondria from one syngen to replicate in the nucleo-cytoplasmic system of another can be obtained. Secondly, the genetic basis of intersyngen mitochondrial protein differences can be studied.

This chapter describes results concerning the interactions between the nucleus and mitochondria of different syngens and the use of intersyngenic variation to determine the inheritance of the mitochondrial enzyme fumarase.

Results

1. Variation in the successful transfer of mitochondria between different syngens.

When erythromycin resistance was transferred from one syngen to another by microinjection of mitochondria prepared from erythromycin-resistant stocks, two classes of result were observed. These were denoted "High" and "Low" efficiency transfers.

TABLE 7:

Efficiency of Transfer of Erythromycin Resistance between Syngens

Expt.No.	Donor Stock	Recipient Stock	Inter-syngen Transfer: No.of cells developing resistance	Inter-syngen Transfer: No. surviving injection	Inter-syngen Transfer: % transfer of resistance	Intra-syngen Transfer (controls): No.of cells developing resistance	Intra-syngen Transfer (controls): No. surviving injection	Intra-syngen Transfer (controls): % transfer of resistance
(i) High Efficiency Transfer								
14	5^{ER}	1^{ES}	18	18	100%	7	9	78%
21, 29	5^{ER}	7^{ES}	32	57	56%	27	33	82%
8, 9	Hybrid cells 5^{ER}m1	1^{ES}	19	26	73%	15	16	94%
(ii) Low Efficiency Transfer								
1, 11	1^{ER}	7^{ES}	2	40	5%	12	14	85%
2, 4, 6	1^{ER}	5^{ES}	4	49	8%	13	20	65%
5	1^{ER}	3^{ES}	0	16	-	13	13	100%
16	1^{ER}	14^{ES}	0	20	-	3	3	(100%)
3	4^{ER}	8^{ES}	0	11	-	9	11	81%
25,30	Hybrid cells 5^{ER}m1	7^{ES}	1	42	2%	15	20	75%
17	1^{ER}M15	5^{ES}	1	22	2%	5	7	70%

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"High" Efficiency transfers were defined as those transfers where more than half of the cells injected acquired erythromycin resistance. This category includes all intra-syngen transfers studied and some inter-syngen transfers. Table 7 (i) shows the intersyngen transfers where at least 50% of the cells injected developed resistance to erythromycin after selection. The time taken for both inter- and intra-syngen cells to develop resistance was the same in these "high" efficiency transfers; about 5 to 6 days. The erythromycin resistance acquired in inter-syngen transfers in these experiments was found to be stable in non-selective medium for at least one year.

"Low" Efficiency transfers were defined as those transfers where less than 10% of the cells injected acquired erythromycin resistance. None of the intra-syngen and only some of the intersyngen transfers fell into this category. Table 7 (ii) shows heterosyngen transfers where the number of cells developing resistance to erythromycin is less than 10% of the total number of cells surviving injection. In contrast, the intra-syngen control transfers injected with the same mitochondrial preparation always gave 65-100% development of resistance.

The erythromycin resistance acquired in "low" efficiency inter-syngen transfers is also stable for at least one year in non-selective media and these cells appear to grow at the normal rate of 3-4 fissions per day, in selective medium. However the time taken to acquire resistance by intersyngen transfer is very much longer (12-29 days) than the time taken by intra-syngen controls (5-6 days) injected in the same experiment, with the same preparation of mitochondria.

These results show that it is possible to transfer erythromycin resistance between genetically isolated groups of Paramecium by injection of mitochondria. It is perhaps significant that successful transfer was

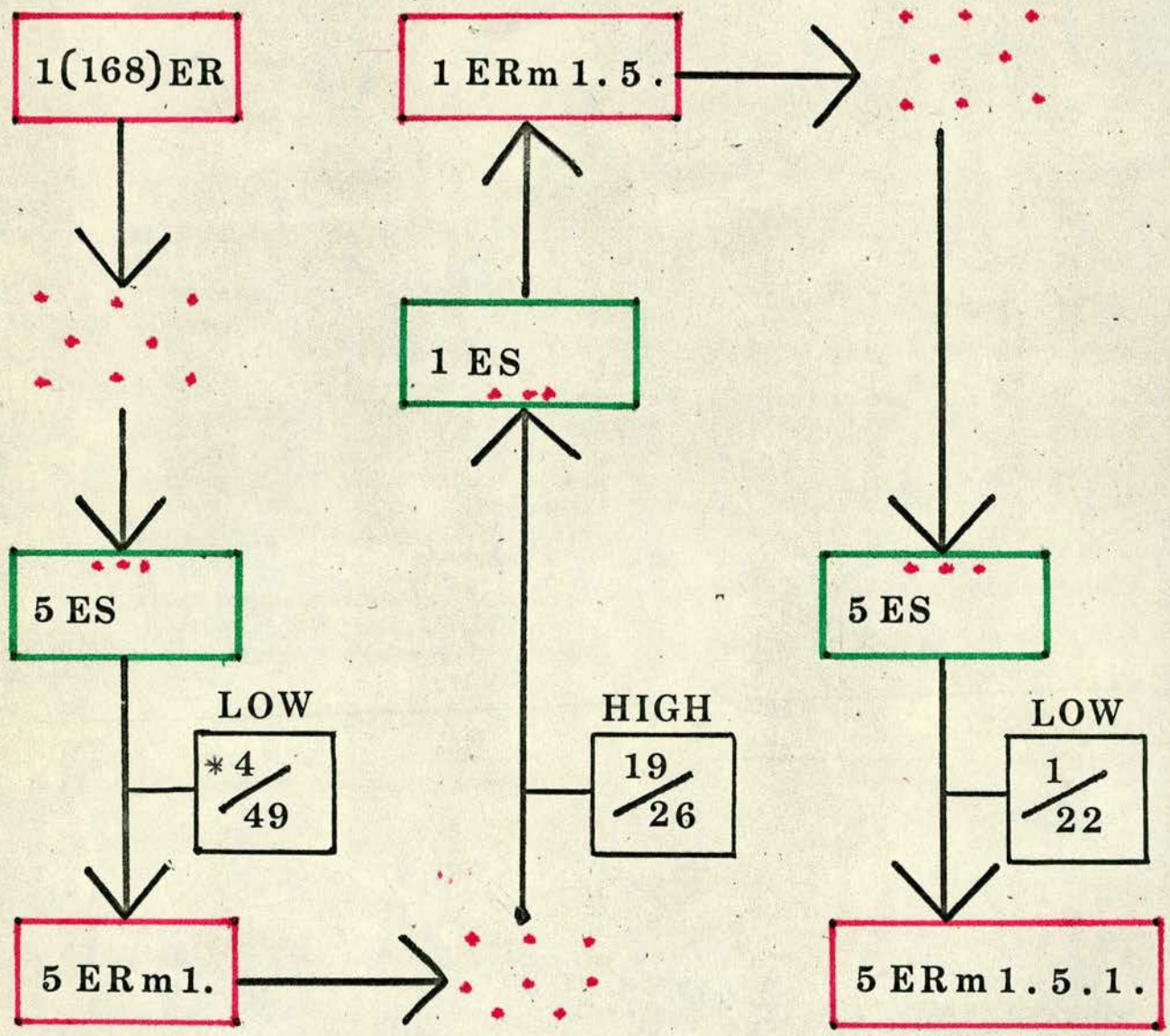

Figure 16. Mitochondrial Transfers Between Syngens 1 and 5.

* $4/_{49}$ - No. of cells developing resistance.
Total No. of cells injected.
(for explanation see text)

only achieved between syngens 1, 5 and 7 (see Table 7). These syngens show morphological and physiological similarities and are considered to be closely related taxonomically. These results do not exclude the possibility that mitochondrial transfer between other syngens and other species of paramecium may be carried out.

All the intersyngen transfer results obtained are shown in Table 7 . However, two particular combinations of results will now be considered in more detail to show more clearly the interesting features of this work.

2. Reciprocal transfer between syngens 1 and 5.

Figure 16 shows the proportion of cells acquiring erythromycin resistance following microinjection with mitochondria originally derived from syngen 1, stock 168 ER_1, and then transferred successively between syngens 1 and 5. The intra-syngen control experiments are not shown. There are two important features of these results.

(i) Transfers from syngen 1, stock 168, to syngen 5, occur with "low" efficiency, and transfers from syngen 5 to syngen 1 occur with "high" efficiency.

(ii) Mitochondria prepared from 5 ER_{m1} give "high" efficiency of transfer when injected directly into syngen 5 intra-syngen control cells (not shown). The same mitochondria give "low" efficiency of transfer when injected into syngen 5 cells after a further passage through syngen 1.

These observations suggest that mitochondria may be modified by growth in syngen 1 cells in such a way that their ability to transfer erythromycin-resistance to syngen 5 cells is lowered.

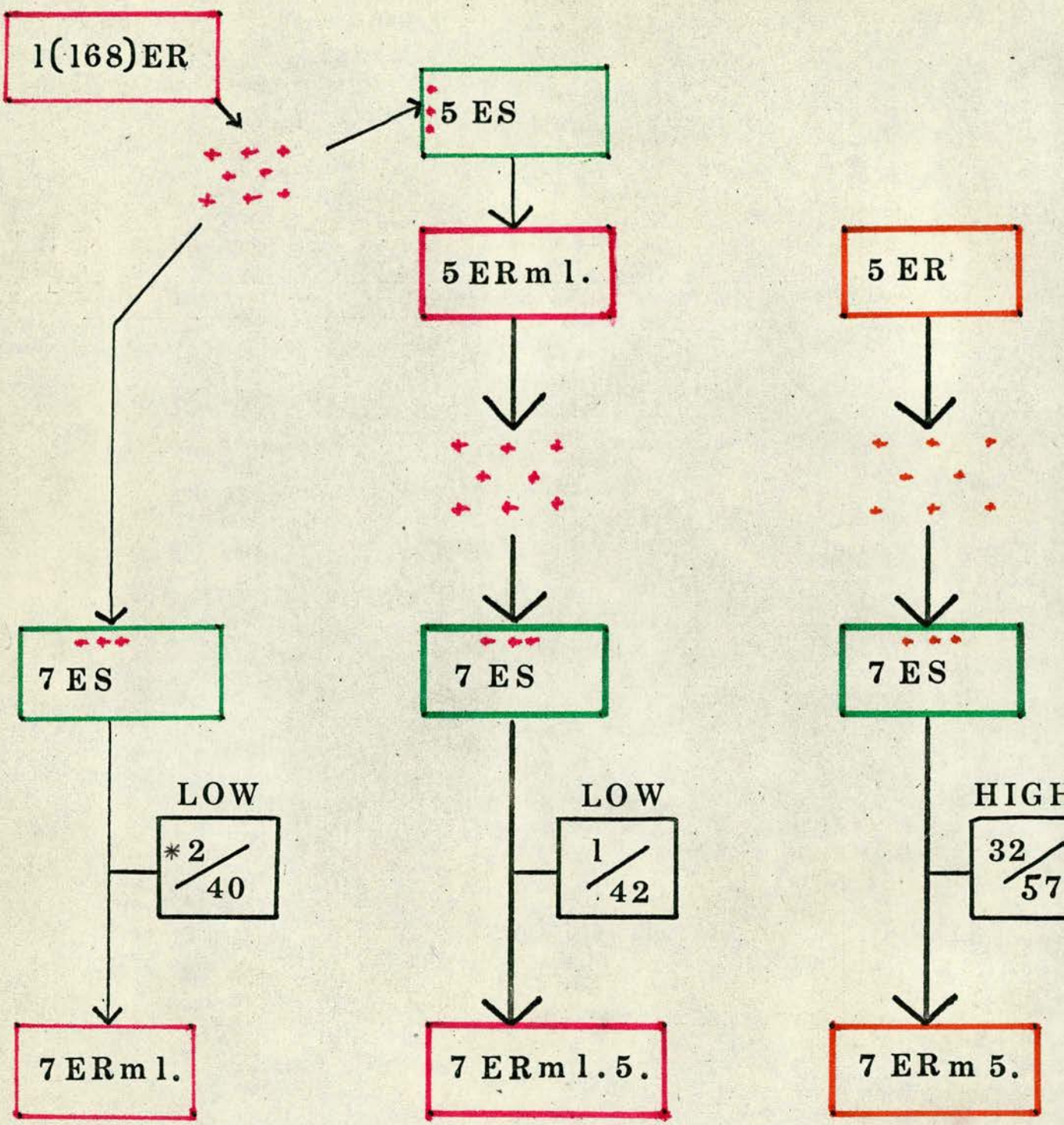

Figure 17. Mitochondrial Transfers Between Syngens 1, 5 and 7.

* $2/_{40}$ - No. of cells developing resistance.
Total No. of cells injected.

(For explanation see text)

3. Transfer of Erythromycin resistance from syngens 1 and 5 to syngen 7.

Figure 17 shows the proportion of syngen 7 cells acquiring erythromycin resistance following microinjection with mitochondria prepared from the erythromycin resistant stocks $5ER_1$ and $5\ ER_{m1}$ is a "hybrid" stock containing erythromycin resistant mitochondria derived from stock 168 ER_1, syngen 1 by microinjection. There are again two important features to be noted.

(i) Erythromycin is transferred with "high" efficiency from 5 ER to 7 ES.

(ii) Erythromycin resistance is transferred with "low" efficiency from 5 ER_{m1} to 7 ES and also from 1 $ER(168_1)$ to 7 ES.

These findings show that in this set of experiments the "low" efficiency transfer of erythromycin resistance from 5 ER_{m1} to 7 ES is probably caused by some mitochondrially inherited character of stock 1(168) ER_1 which is not modified by growth in syngen 5 cells.

4. The Genetic Control of the Mitochondrial Enzyme Fumarase

Syngen 7 sensitive cells were microinjected with mitochondria prepared from syngen 1 erythromycin resistant cells and after 21 days incubation in selective medium, 2 out of a total of 40 microinjected syngen 7 sensitive cells became resistant (Table 7). The transfer of erythromycin resistance implies that the mitochondrial genome of syngen 1 may be introduced and replicated in a cell containing a syngen 7 nucleus. This "hybrid" cell may be used to locate the genetic information coding for any character which shows variation between the two "parental" stocks (see Fig.18). (overleaf).

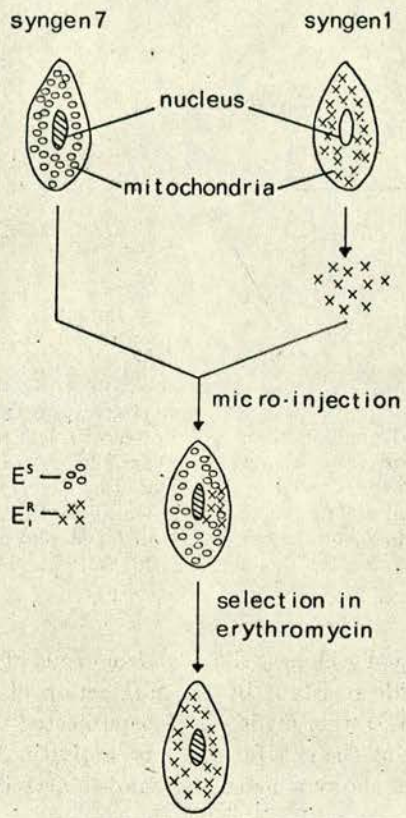

Figure 18. Transfer of mitochondria by micro-injection between different syngens, showing the origin of the syngen 1/7 "hybrid".

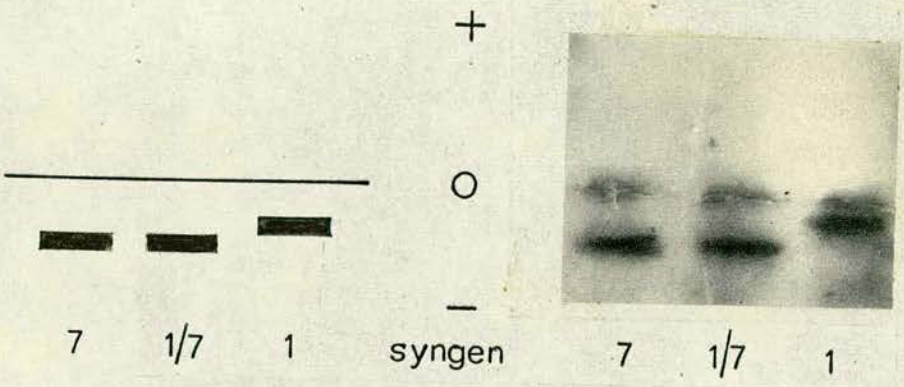

Figure 19. Starch gel zymogram of funarase, showing electrophoresis of extracts from syngen 1 ($168E_1^R$), syngen 7 (227) and syngen 7 cells micro-injected with syngen 1 erythromycin resistant mitochondria (7/1). Electrophoresis was carried out for 3 hours at 0°C in a 0.01 M phosphate-citrate buffer pH = 5.5. Enzyme activity was localised by overlaying the gel with 30 mls of staining solution (3.9 mg/ml sodium fumarate, 0.25 mg/ml NAD, 0.2 mg/ml MTT-tetrazolium, 0.02 mg/ml PMS and 1 i.u. malic dehydrogenase in 0.1 M phosphate buffer pH = 7.4) and incubating at 35°C.

It is known that syngens 1 and 7 differ with regard to the electrophoretic mobility of their mitochondrial fumerase (Tait, 1970). Extracts from cells of stocks 1 ER, 7 ES (parental) and 7 $ER_{m\ 1}$ (hybrid) were made and characterised by starch gel electrophoresis. Fig.19 shows the bands of fumarase activity from these stocks. The hybrid 7 $ER_{m\ 1}$ shows a band of fumarase activity identical in mobility with that shown by 7 ES and different from that shown by 1 ER.

The result shows that fumerase is not controlled by the syngen 1 erythromycin resistant mitochondrial genome and by inference must be controlled by the syngen 7 nuclear genome.

Discussion

1. Variation in Efficiency of Resistance Transfer

Results have been presented which show that, in some intersyngen transfers, erythromycin resistance is transferred to only a small proportion of those cells injected. Most of the intra-syngen cells injected with the same preparation of mitochondria do develop resistance. In addition, the intersyngen cells take considerably longer to develop resistance than do intra-syngen cells. A priori, it might be argued that mitochondria could behave in two ways after transfer to a new stock. Either the introduced organelles are able to replicate in the new stock or, because of some genetic incompatability with the strange nuclear products, they are not.

However, these results suggest that, in some cases, more complex processes determine whether an introduced mitochondria will grow within the cytoplasm of a foreign syngen. While the nature of these complex processes is still obscure, it is clear that nuclear and mitochondrially inherited factors can cause "low" efficiency transfer of mitochondria between syngens. Consideration of reciprocal transfers of erythromycin resistance between syngens 1 and 5 has shown that replication of mitochondria in syngen 1 (168) modifiesthese mitochondria in such a way that "low" efficiency transfer is observed on retransfer to syngen 5. This modification causing low efficiency transfer could take at least three forms.

(i) It is possible that the mitochondrial DNA is modified in some way by enzymes of the host cell. This system might be analogous with the modification and restriction system well documented in bacterial systems (Arber, 1971). "Low" efficiency transfers could be caused by the degradation of the unmodified introduced DNA by "host" restriction enzymes. There would be a finite probability that an introduced genome might be modified before restriction, in which case the "host" cell could become erythromycin resistant by replication of the newly modified DNA conferring resistance.

(ii) It is possible that the structure of a mitochondria from one syngen may be "incompatible" in some way with the nuclear cytoplasmic systems of another syngen. No replication of the newly introduced mitochondria could occur until the "incompatable" units had been replaced by a new paracrystaline structure. Once the introduced genome conferring resistance was associated with a structure

compatable with the mitochondrial systems of the host cell, replication of this genome could occur.

(iii) Recombination between the introduced DNA and the host mitochondrial DNA is another possibility. However, the results under discussion are unlikely to be explained by recombination as this requires the rate of mitochondrial recombination to be very much higher in syngen 1 than in syngen 5. This seems to be unlikely. Furthermore repeated attempts to obtain recombination between erythromycin and chloramphenicol resistance markers in syngen 1 (Beale et al, 1972) and syngen 4 (Adoutte and Beisson, 1972) have failed to produce any convincing results.

However, it is not yet possible to decide between modification of the mitochondrial DNA itself and modification or replacement of elements of the mitochondrial structure as an explanation of these findings.

Consideration of the results of transferring two different classes of mitochondria from syngen 5 to syngen 7 has shown that in some cases a mitochondrially inherited character can cause "low" efficiency transfer between syngens. This character is likely to be of at least one of two basic types.

(i) It could be a physical site on the mitochondrial DNA related to the replication of physiological functions of the organelle. Before the introduced resistant genome could be expressed, a new type of interaction between this DNA and the host mitochondrial system would have to occur. This might involve the formation of a new paracrystaline structure "compatible" with the attachment sites on the DNA.

(ii) Alternatively, any one of the products of mitochondrial DNA which interact with the "host" mitochondrial components to form a self organising paracrystaline structure could cause the mitochondrially inhibited "low" efficiency transfer. In order for the introduced mitochondria to replicate, a new self-organising structure "compatible" with the "host" mitochondrial compoents must occur.

Any explanation of "low" efficiency of transfer must account for the process by which mitochondria acquire the ability to replicate in a cell in which they were previously unable to grow. This ability to replicate is not lost until the mitochondria are retransferred into a cell of another syngen. Much of the current thought concerning the structure of organelle membranes evokes the concept of paracrystaline structures whose nature and composition is determined in part by the pre-existing structure (Green et al. 1971; Benson et al., 1971; Mahler et al., 1971). A paracrystaline structure has the property of being able to "switch" from one stable form to another stable form in the manner required to explain the phenomenon of "low" efficiency transfer. Certainly, the evidence presented in this chapter does not conflict with the hypothesis that some mitochondrial characters may be determined by pre-existing epigenetic structures ~~and not only~~ as well as by the nuclear and mitochondrial DNAs.

2. The Genetic Control of the mitochondrial enzyme fumarase

Certain soluble mitochondria enzymes have been shown to be controlled by nuclear genes in both Paramecium (Tait, 1968, 1970) and other organisms (Munkres et al., 1965; Munkres and Woodward, 1967; Davidson and Corner, 1967; Longo and Scandalios, 1969; Davidson et al., 1970) using within-species genetic analysis. The

observations presented in part 2 of the results provide further evidence for the idea that all the soluble mitochondrial enzymes are controlled by the nuclear genome. Because intra-syngen variation of fumerase with respect to electrophoretic mobility has not been found, intersyngenic hybrid cells produced by microinjection were used to determine the nature of the genetic control of fumerase. The results presented strongly suggest that fumerase is coded for by the nuclear genome.

In summary, the results obtained so far show two classes of interaction causing "low" efficiency transfer. One class, illustrated by reciprocal transfer between syngens 1 and 5, is caused by a modification of the mitochondria by the nucleus such that the organelle becomes altered in its ability to transmit erythromycin resistance following injection.

The second class of interaction illustrated by transfers between 1, 5 and 7 shows that the origin of the mitochondrial genome is also important in controlling transmission or erythromycin resistance from one syngen to another. By analysis of intersyngen "hybrid" cells it has been shown that the mitochondrial enzyme fumerase is coded for by nuclear genes.

GENERAL CONCLUSION AND PROSPECTS

The technique of microinjection has often proved a useful way of investigating the biology of the cell and its components. The work described in this study uses the technique of microinjection to investigate directly features of mitochondrial genetics in Paramecium aurelia. Initially this technique was used to show that erythromycin resistance was located in the mitochondria and that acquisition of erythromycin resistance by a sensitive cell after microinjection was the result of selection for the erythromycin resistant mitochondria introduced.

Perhaps the most important advantage of microinjection is that it makes possible transfer of mitochondria across natural genetic barriers. This has two important consequences. Firstly, it allows investigation of the ability of mitochondria of one syngen to grow in the cytoplasm of other syngens. From this type of work both nuclear and mitochondrial factors have been found to modify the way in which mitochondria behave after transfer to a different syngen. In addition, some evidence was found to support the idea that the pre-existing organization of the mitochondrial components also affected the ability of mitochondria to grow in cells of a different syngen. Secondly, it allows the production of a cell containing the mitochondria of one syngen and the nucleus of another.

The exact nature and function of the proteins coded by the mitochondrial DNA still remains one of the greatest problems in mitochondrial genetics. As stated earlier, the definitive

most probably

identification of a protein coded by the mitochondrial DNA requires the correlation of changes in the mitochondrial DNA with changes in that protein. There are three possible approaches to this problem

(1) The study of proteins altered by cytoplasmic mutations. The results so far obtained by this approach have proved equivocal. In Paramecium, Beale et al (1971) have reported that cytoplasmic erythromycin-resistance altered the protein composition of mitochondrial ribosomes, but the nature of this alteration has proved difficult to define. Similar results were obtained in yeast, but further investigation showed that this apparent ribosomal protein difference might have been an artefact caused by the techniques used (Borst, private communication).

(2) The study of mitochondrial proteins showing maternal inheritance. Dawid (1972) has shown by hybridization studies that mitochondrial DNA is maternally inherited in crosses between two species of Xenopus. However, any demonstration that a protein is coded for by the mitochondrial DNA would require extensive back crossing to the parental species, a procedure made difficult by the partial or total sterility of this inter-species hybrid.

(3) The study of artificially produced "hybrid" cells containing the mitochondria of one species and the nucleus of another. The production of this type of cell by microinjection has been discussed at length in this study. It is known that many soluble mitochondrial enzymes vary between syngens (Tait, 1968, 1970a/b) and

there is no reason to suppose that insoluble mitochondrial proteins do not vary in the same way. The careful analysis of the protein products of mitochondrial protein synthesis by disc-gel and immuno-electrophoresis should allow identification of those proteins which vary between syngens. Similar analysis of the mitochondrial protein of the "hybrid" cell would allow the location of the information coding for the variant protein in either the mitochondrial or the nuclear genome.

The techniques just described will be exploited in the future in an attempt to identify proteins coded by the mitochondrial DNA. It is now evident that only by the simultaneous use of the techniques both of genetics and biochemistry will the fascinating problems of mitochondrial genetics be solved.

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PUBLICATIONS

1. Observations on two mitochondrial phenotypes in single paramecium cells. J.K.C. Knowles, Exptl. Cell Res. 70 (1972), 223.

2. Mitochondrial Genetics in *Paramecium*. G.H. Beale, J.K.C. Knowles, A. Tait. Nature 235 (1972), 396.

3. A New Method for Studying the Genetic Control of Specific Mitochondrial Proteins in *Paramecium aurelia*. J.K.C. Knowles and A. Tait. Molec.Gen.Genet. 117 (1972), 53.

The results obtained by the author and described in these publications were part of the research presented in this thesis.

Observations on two mitochondrial phenotypes in single paramecium cells

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In yeast, evidence has been published [1, 2] indicating that resistance to erythromycin and chloramphenicol is controlled by mitochondrial DNA, and in *Paramecium aurelia* Beale et al. [3] and Adoutte & Beisson [4] have also shown that resistance to the same drugs is controlled by extranuclear genetic factors, most probably located within the mitochondria. These genetical studies have all been based on observations on the behaviour of whole cells. In the work to be described here, the effect of erythromycin on individual mitochondria as seen by electron microscopy will be considered.

Paramecia collected from nature are sensitive to erythromycin, i.e. when they are placed in bacterized grass medium [5] containing the drug at a concentration of 0.25 mg/ml, and incubated at 25°C, cell division is blocked after one or two residual fissions. However, the organisms are not killed for some 2 to 4 weeks, but swim slowly and become black and thin. Drug-resistant mutants have been obtained [3, 4] and these grow and

Table 1. *Relative proportions of the different types of mitochondria in transforming cells*

Cell	Total of mitochondrial sections counted	Mitochondrial types		
		% resistant	% sensitive	% "intermediate"
A	394	77	22	1
B	382	78	21	1
C	638	73	26	1
D	303	81	18	1
E	184	86	13.5	0.5

cells, entire sections from five cells, taken from three separate transformation experiments, were photographed and the counts from these photographs are shown in table 1.

In view of the similarity in the proportions of the three mitochondrial types in the five cells, it is of interest to point out that these were all selected on the basis of their pear-shape and speed of movement of the paramecia, from which it was concluded that they were in the process of transforming from the sensitive to the resistant state.

The main conclusion from these provisional observations, based on study of single cells, is that within a single paramecium two (and often three) types of mitochondria can coexist in a common cytoplasmic environment, thus strengthening the hypothesis that mitochondria have a partial genetic autonomy.

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The author is a MRC Post-Graduate Research Student.

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